Resistance to Drug-induced Apoptosis in T-cell Acute Lymphoblastic Leukemia

LEUNG Kam Tong

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Thesis Committee:

Prof. Samuel S. M. Sun (Supervisor)

Prof. Peter P. K. Cheung (Internal Examiner)

Prof. Anthony H. Y. Chung (Internal Examiner)

Prof. George S. W. Tsao (External Examiner)

Abstract

T-cell acute lymphoblastic leukemia (T-ALL) is a malignant disease of the bone marrow in which early lymphoid T-precursor cells proliferate and replace normal hematopoietic cells. Despite there has been remarkable progress in the development of effective treatments against T-ALL, 20% of the patients still exhibit poor response to the current chemotherapeutic regimens and the cause of treatment failure in these patients is still largely unknown. Defects in the apoptotic pathway may account for chemoresistance in at least some T-ALL patients since chemotherapy acts primarily by inducing apoptosis. Therefore, better understanding of the mechanisms that cause resistance to apoptosis in T-ALL cells may provide insights into the development of novel strategies for the treatment of T-ALL patients with unfavorable response to anticancer drugs.

By studying the cellular responses to an apoptogenic drug, etoposide, we identified an apoptosis-resistant T-ALL cell line, Sup-T1. We showed that Sup-T1 cells were deficient in etoposide-induced activation of caspases and mitochondrial cytochrome *c* release. However, addition of exogenous cytochrome *c* in cell-free apoptosis reactions induced prominent caspase-3 activation, indicating that the chemoresistance observed in Sup-T1 cells is due to its insusceptibility to the drug-induced mitochondrial alterations. Analysis of expression of the Bcl-2 family proteins revealed that the levels of Bax, Bcl-2 and Bim in Sup-T1 cells were differed from etoposide-sensitive T-ALL cell lines.

Transient transfection of Bim_{EL} into Sup-T1 cells significantly restored their sensitivity to the etoposide-induced apoptosis. This provides direct evidence showing that the expression level of Bim is an important determinant for the sensitivity of T-ALL Sup-T1 cells to the etoposide-induced apoptosis.

Further experiments revealed that the lack of Bim_{EL} expression in Sup-T1 cells was due to the rapid degradation of newly-synthesized Bim_{EL} by the proteosomal pathway, as treatment of Sup-T1 cells with a proteosome inhibitor significantly restored the protein level of Bim_{EL}. In addition, we showed that constitutive activation of the JNK pathway in Sup-T1 cells was responsible for promoting Bim_{EL} phosphorylation, and this serves as a signal targeting Bim_{EL} to the proteosome for degradation. Altogether, our findings represent a possible mechanism of chemoresistance in T-ALL cells, and provide new insights into the regulation of Bim and the role of JNK pathway in cell survival. It is expected that these findings will help to develop novel, target-based strategies for the treatment of resistant T-ALL.

摘要

T 細胞急性白血病對藥物誘導凋亡的抵抗

T 細胞急性白血病 (T-ALL) 是一種骨髓的惡性疾病，在 T-ALL 病患者的骨髓內，T 前體細胞不斷增殖，並取代原有的造血細胞。雖然現在治療 T-ALL 的成功率已大大提高，但是有百分之二十的病人仍對現有藥物的反應甚差，而且，我們仍未知道治療失敗的原因。由於化療藥物的主要功能是誘導細胞凋亡，所以我們相信某部分 T-ALL 病患者出現抗藥性，是因爲他們 T-ALL 細胞內的凋亡訊息傳導路徑出現毛病。如果知道 T-ALL 細胞是怎樣抵抗藥物誘導的凋亡，這可能會幫助我們發展新的策略去治療抗藥性的 T-ALL 病人。

透過探討 T-ALL 細胞對抗癌藥 etoposide 的反應，我們發現了一條抗凋亡的 T-ALL 細胞線 Sup-T1，並以此作爲研究抗藥機制的細胞模型。研究結果顯示，Sup-T1 細胞可以抵抗 etoposide 誘導的天冬氨酸特異性半胱氨酸蛋白酶 (caspase) 的活化以及粒線體細胞色素 C 的釋放，但是，在無細胞凋亡反應中加入外來的細胞色素 C，Sup-T1 的 caspase-3 便明顯活化了，這代表 Sup-T1 細胞出現抗藥性是因爲它的粒線體不能在藥物刺激時產生變化。通過分析 Bcl-2 家族蛋白的表達，我們發現 Sup-T1 細胞內 Bax、Bcl-2 及 Bim 的表達程度，有別於其他對 etoposide 敏感的 T-ALL 細胞線，而當我們把 Bim_{EL} 過渡性轉染到 Sup-T1 細胞內，它對 etoposide 的敏感度便得以恢復，這證明了 Bim 的表達程度是 Sup-T1 細胞對 etoposide 敏感度的決定因素。

最後，我們還探討了 Bim_{EL} 在 Sup-T1 細胞內流失的原因。研究結果顯示在 Sup-T1

細胞內，新製造的 Bim_{EL} 蛋白會被蛋白質解體迅速裂解，因爲在經蛋白質解體抑制物處理的 Sup-T1 細胞內，Bim_{EL} 蛋白的表達程度顯著回升。另外，我們發現 Sup-T1 細胞內的 JNK 路徑是持續活化的，活化 JNK 會把 Bim_{EL} 磷酸化，這樣，磷酸化 Bim_{EL} 便會被帶往蛋白質解體進行裂解。總括而言，我們的數據顯示了 T-ALL 細胞的一種新抗藥機制，我們還更深入認識了 Bim 的調控以及 JNK 路徑在細胞抗凋亡上所扮演的角色，我們預期這些發現能夠幫助發展新的靶向策略去治療抗藥性的 T-ALL 病人。

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ALL	Acute lymphoblastic leukemia
Apaf-1	Apoptotic protease activating factor-1
B-ALL	B-cell acute lymphoblastic leukemia
BH	Bcl-2 homology
Bim	Bcl-2 interacting mediator of cell death
Caspases	Cysteine aspartyl-specific proteases
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CIAP	Calf intestinal alkaline phosphatase
Cyt *c*	Cytochrome *c*
dATP	Deoxyadenosine triphosphate
DISC	Death-induced signalling complex
DTT	Dithiothreitol
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain
FLIPs	FADD-like interleukin-1β-converting enzyme-like protease-inhibitory proteins
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HSPs	Heat shock proteins
IAP	Inhibitor of apoptosis
ICAD	Inhibitor of the caspase-activated deoxyribonuclease
IκB	Inhibitor of nuclear factor-kappaB
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MRP-3	Multidrug resistance-associated protein 3

NF-κB	Nuclear factor-kappaB
PBS	Phosphate-buffered saline
PI	Propidium iodide
Pre-B-ALL	Precursor B-cell acute lymphoblastic leukemia
RNase A	Ribonuclease A
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium docecyl sulfate-polyacrylamide gel electrophoresis
TMRE	Tetramethylrhodamine ethyl ester
TNF	Tumor necrosis factor
z-VAD-fmk	N-benzyloxycarbonyl-Val-Ala-aspartyl-fluoromethyl ketone

Chapter 1 General Introduction

1.1 Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is a rapidly progressing disease results from the neoplastic proliferation of immature lymphoid cells called lymphoblasts. Normally, blasts constitute 5% or less of the healthy bone marrow. In ALL, however, these blasts remain abnormally immature and multiply continuously, eventually constituting between 30% to 100% of the bone marrow. The expanded lymphoblast population fills up the bone marrow and prevents the production of healthy red cells, platelets, and mature leukocytes. Consequently, anemia, thrombocytopenia, and neutropenia develop, which, if untreated, become lethal. ALL is the most common type of malignancy in children and constitutes 80% of the leukemia up to the age of 15. In adults, ALL is relatively less common and constitutes 15-20% of all cases of acute leukemia (Berg *et al.*, 2000). Immunophenotyping of ALL blast cells is the major method for classifying ALL subtypes. By detection of lineage-specific antigens, ALL blast cells can be subdivided into B-lineage and T-lineage acute lymphoblastic leukemia. ALL blast cells that express the B-lineage markers can also be subclassified into mature B-cell acute lymphoblastic leukemia (B-ALL) and precursor B-cell acute lymphoblastic leukemia (pre-B-ALL). Whereas, those express T-lineage markers are generally termed as T-cell acute

lymphoblastic leukemia (T-ALL) (Foon *et al.*, 1980). Approximately 15% of children and adults with ALL are diagnosed as T-ALL, and the rest are of the B-lineage (Pui, 2005).

1.2 T-cell acute lymphoblastic leukemia

1.2.1 Chemotherapy

Chemotherapy is the primary treatment for T-ALL. It is generally divided into three phases: induction, intensification, and maintenance. Many patients also receive treatment called intrathecal chemotherapy to prevent leukemia from spreading to the central nervous system.

1.2.1.1 Induction therapy

Induction therapy of T-ALL aims to induce complete remission. To achieve complete remission, the leukemic burden has to be reduced by 99%. Typically, 4 to 5 drugs are used for remission induction. The current regimens contain vincristine, steroids, and daunorubicin plus either asparaginase or cyclophosphamide. Similar outcomes were evidenced from different induction therapy programs, with approximately 80-90% of the patients achieve a complete remission (Larson, 2003).

1.2.1.2 Intensification therapy

Intensification therapy is designed to eradicate the rapidly proliferating blast cells that are responsible for early relapse. Drugs given for this purpose are usually antimetabolites such as methotrexate. The need for intensive intensification therapy to achieve a cure, unlike that of remission induction therapy, is controversial. In randomized trials, increasing doses of chemotherapy during intensification therapy did not improve the results (Ribeiro and Pui, 2005).

1.2.1.3 Maintenance therapy

For maintenance therapy, T-ALL patients receive a prolonged period of treatment with low doses of chemotherapeutic drugs. The period of maintenance therapy is depended on the intensity and success of the previous phases of chemotherapy. Typically, 1 to 3 years of 6-mercaptopurine and methotrexate are often used in this phase of therapy (Larson, 2003).

1.2.2 Chemoresistance in T-ALL

Previous studies have shown that patients with T-ALL have poorer treatment success than those with B-lineage ALL. Henze *et al.* reported that the 9-year probability of continuous complete remission were 9% ± 9% and 41% ± 5%, for patients with T-lineage ALL and non-T-lineage ALL, respectively (Henze *et al.*, 1981). Although treatment outcome in newly diagnosed T-ALL has improved significantly in several recent clinical trials (Goldberg *et al.*, 2003; Pui *et al.*, 2004), higher rates of induction failure, induction death and early relapse are consistently found in T-ALL patients. These clinical data suggest that patients with T-ALL may be more resistant to conventional chemotherapeutic agents.

Owing to the remarkable progress in the treatment strategies, T-ALL is now curable with chemotherapy in over 80% of patients. However, the cause of treatment failure in the remaining patients is still largely unknown. Only a few reports have highlighted the underlying mechanisms. The cause of glucocorticoids resistance is controversial. Quddus *et al.* reported that glucocorticoids receptor levels in leukemic cell could not be used to predict the treatment outcome (Quddus *et al.*, 1985). However, Costlow *et al.* reported that lower glucocorticoids receptor levels were correlated with unfavorable presenting features (Costlow *et al.*, 1982). More recently, several differentially expressed genes have been identified in ALL cells with resistance to prednisone, vincristine and daunorubicin, whose pattern of expression is related to treatment outcome (Holleman *et al.*, 2004).

However, it is still not known whether modulation of the proteins encoded by these genes will enhance treatment efficacy in patients with drug-resistant ALL.

1.3 Apoptosis and chemoresistance

Cells have an inherent suicidal program called apoptosis. Since chemotherapeutic drugs act primarily by inducing apoptosis, defects in the apoptotic machinery can make cancer cells resistant to therapy. Thus, understanding of how tumor cells resist apoptosis might lead to the development of novel therapeutics that target at restoring apoptosis sensitivity (Igney and Krammer, 2002).

1.3.1 Initiation, execution and regulation of apoptosis

1.3.1.1 Initiation of apoptosis

Apoptosis can be initiated by two distinct pathways. The extrinsic pathway is mediated by death receptors on the cell surface. The intrinsic pathway is mediated by mitochondria (Krammer, 1999). Both pathways ultimately converge on the activation of cysteine aspartyl-specific proteases (caspases). Activated caspases in turn cleave cellular death substrates, resulting in the biochemical and morphological changes that are hallmarks of apoptosis.

The extrinsic pathway is activated by the engagement of death receptors on the cell surface. Binding of death ligand such as FasL or tumor necrosis factor (TNF) to their respective receptors recruits Fas-associated death domain (FADD) and pro-caspase-8 to form a complex known as death-induced signalling complex (DISC). Oligomerization of pro-caspase-8 in the DISC autoactivates and releases mature caspase-8, which functions as an initiator caspase (Salvesen and Dixit, 1999). Depending on the cell type, active caspase-8 induces apoptosis by two different signalling pathways. In type I cells, large amount of active caspase-8 formed at the DISC induces apoptosis directly. In type II cells, the small amount of active caspase-8 is insufficient to initiate apoptosis directly. To overcome the problem, caspase-8 cleaves the BH3-only protein Bid to generate an active fragment, tBid, which activates the intrinsic pathway (Scaffidi *et al.*, 1998).

The intrinsic pathway, on the other hand, is initiated by permeabilization of the mitochondrial membrane. Loss of the mitochondrial membrane potential induces leakage of apoptogenic molecules including cytochrome *c*, SMAC/DIABLO and apoptosis inducing factor into the cytosol (Zamzami and Kroemer, 2001). In the cytosol, cytochrome *c* induces the oligomerization of Apaf-1 (apoptotic protease activating factor-1) in the presence of dATP (deoxyadenosine triphosphate). Apaf-1 oligomers in turn recruit pro-caspase-9 to form a complex known as apoptosome. Within the apoptosome, cleavage of pro-caspase-9 yields active caspase-9 which functions as an

initiator caspase.

1.3.1.2 Execution of apoptosis

Activated initiator caspases process and activate the downstream effector caspases including caspase-3, -6 and -7. Active effector caspases, in turn, cleave cellular death substrates, resulting in the biochemical and morphological alterations of apoptosis (Earnshaw *et al.*, 1999). Caspase substrates in cytoskeleton include actin, β-catenin, and gelsolin. Cleavage of these proteins leads to the formation of apoptotic bodies. Cleavage of nuclear lamins such as lamin A will result in nuclear shrinkage. DNA fragmentation is associated with the cleavage of ICAD (inhibitor of the caspase-activated deoxyribonuclease). Moreover, effector caspases also cleave proteins involved in DNA repair, signal transduction pathways, and cell cycle regulation (Earnshaw *et al.*, 1999).

1.3.1.3 Regulation of apoptosis

Apoptosis is stringently controlled and can be regulated by different classes of molecules at various levels. Apoptosis can be regulated at the mitochondrial level by members of the Bcl-2 family proteins. The human Bcl-2 family comprises over 20 molecules that can be classified functionally into anti-apoptotic and pro-apoptotic members (Cory and Adams, 2002). These proteins contain 1 to 4 conserved Bcl-2

homology (BH) domains. Examples of the anti-apoptotic members are Bcl-2, Bcl-x_L, Bcl-w, and Mcl-1. They are localized to the mitochondrial outer membrane where they prevent the release of mitochondrial cytochrome *c* into the cytosol. The pro-apoptotic members can be subdivided into 2 subfamilies. The Bax subfamily comprises Bax, Bak, and Bok. They share sequence homology among the BH1, BH2 and BH3 domains. The BH3-only subfamily comprises Bad, Bid, Noxa, Puma, Bik, Bim, Bmf, and Nip-3. BH3-only proteins act as sensors of cellular damage and initiate the death process. Upon apoptotic stimuli, these proteins undergo post-translational modifications and redistribute to mitochondria. For example, Bid is cleaved and activated by caspase-8 and Bim is detached from dynein motor complex (Puthalakath and Strasser, 2002). BH3-only proteins, in turn, induce mitochondrial permeabilization either by inhibiting the anti-apoptotic proteins or by activating pro-apoptotic proteins of the Bax subfamily (Bouillet and Strasser, 2002).

Apoptosis can also be regulated at the level of caspase activation by two classes of proteins. The inhibitor of apoptosis (IAP) proteins is originally identified because of their ability to inhibit apoptosis in the baculovirus-infected cells. Nine IAP family members including XIAP, c-IAP1, c-IAP2, NAIP, MLIAP, ILP2, livin, apollon and survivin have been identified in human cells. All of these proteins possess at least one domain known as baculoviral IAP repeat, which is essential for their anti-apoptotic function. Unlike the

Bcl-2 family of proteins, IAPs directly bind to and inhibit caspase-3, -7, and -9 (Salvesen and Duckett, 2002).

FLIPs (FADD-like interleukin-1β-converting enzyme-like protease-inhibitory proteins) represent another class of proteins that can regulate apoptosis at the level of caspase activation. However, unlike IAPs, they inhibit apoptosis at the level of death receptors (Krueger *et al.*, 2001). FLIPs contain an inactive caspase-8-like catalytic domain at the C-terminus and they are recruited to the DISC during apoptosis, thus preventing procaspase-8 recruitment and activation.

1.3.2 Mechanisms of resistance to apoptosis

1.3.2.1 Overexpression of pro-survival proteins

Numerous studies have shown that overexpression of anti-apoptotic proteins can render tumor cells resistant to apoptosis. Bcl-2 overexpression was initially described in follicular lymphomas as a result of a t(14;18) translocation to the immunoglobulin locus (Tsujimoto *et al.*, 1985). Enhanced expression of Bcl-2 prevents apoptosis induced by chemotherapeutic drugs, including alkylating agents and topoisomerase inhibitors (Kamesaki *et al.*, 1993; Walton *et al.*, 1993). Moreover, studies in clinical samples have revealed a correlation between high expression of Bcl-2 and poor prognosis in a number of tumors, including acute myelogenous leukemia, acute lymphocytic leukemia,

non-Hodgkin's lymphomas, and prostate cancer (Reed, 1999).

Other anti-apoptotic Bcl-2 family members may also involve in determining sensitivity of tumor cells to apoptosis. For example, high Bcl-x_L levels are correlated with poorer prognosis in patients with lymphomas (Bairey *et al.*, 1999), squamous cell carcinoma (Aebersold *et al.*, 2001), soft-tissue sarcomas (Kohler *et al.*, 2002), and myelodysplastic syndrome (Boudard *et al.*, 2002). Moreover, overexpression of Mcl-1 was found to cause a prolongation of cell viability under various conditions that cause apoptotic cell death (Zhou *et al.*, 1997).

High levels of FLIP are evidenced in melanomas (Irmler *et al.*, 1997) and gastric adenocarcinoma (Zhou *et al.*, 2004). Expression of these proteins blocks apoptosis induction at the level of the death receptors. Thus, FLIP expression cannot inhibit cell death induced by granzyme, chemotherapeutic drugs or γ-irradiation (Kataoka *et al*, 1998).

Recent evidence has suggested proteins of the IAP family are key determinant in chemoresistance in cancer cells. Downregulation of XIAP expression was shown to induce apoptosis and enhance chemotherapeutic sensitivity in human gastric cancer (Tong *et al.*, 2005). c-IAP2 overexpression is a frequent event in pancreatic cancer and could therefore potentially mediate resistance in neoplastic pancreatic cancer cells (Esposito *et al.*, 2006). In neuroblastoma, the expression of survivin correlates with a more aggressive

and unfavourable disease (Adida *et al.*, 1998).

Cellular response to various chemotherapeutic drugs can also be determined by the Akt survival pathway. Transfection of constitutively active Akt in lung cancer cells hampers topotecan-induced apoptosis (Nakashio *et al.*, 2000). Fibroblasts overexpressing Akt are resistant to the staurosporine-induced apoptosis (Tang *et al.*, 2001). PTEN, the antagonist of Akt pathway, is frequently deleted in tumors, and a significant rate of PTEN mutations can be found in various cancer types (Steck *et al.*, 1997; Suzuki *et al.*, 1998). PTEN-deficient tumor cell lines and tumors derived from PTEN-knockout mice are resistant to apoptotic stimuli (Wu *et al.*, 2003).

1.3.2.2 Downregulation and mutation of pro-apoptotic proteins

Besides overexpression of anti-apoptotic genes, tumors can acquire apoptosis resistance by downregulating or mutating pro-apoptotic molecules. In colon cancer and hematopoietic malignancies, the pro-apoptotic Bcl-2 family member Bax is mutated (Rampino *et al.*, 1997; Meijerink *et al.*, 1998). Loss of expression by frameshift mutations and loss of function by mutations in the BH domains are the most common forms of mutations in these tumors. Tumor cell lines with frameshift mutations are more resistant to apoptosis. Reduced Bax expression is associated with a poor response rate to chemotherapy and shorter survival (Krajewski *et al.*, 1995). In a transgenic mice model,

induction of Bax expression by p53 resulted in slow tumor growth and a high percentage of apoptotic cells (Krajewski *et al.*, 1995). In Bax-deficient mice, however, tumor growth was accelerated and apoptosis was diminished (Yin *et al.*, 1997).

Tumor cells have developed various strategies to prevent the induction of apoptosome-driven apoptosis. Apaf-1 is an adaptor molecule that binds to and promotes pro-caspase-9 activation in the presence of cytochrome *c*. The Apaf-1 gene is frequently hypermethylated and therefore silenced in melanoma cells (Soengas *et al.*, 2001) and human leukemia (Fu *et al.*, 2003). Apaf-1-negative melanomas fail to respond to chemotherapy (Soengas *et al.*, 2001). Recently, Apaf-1 was found to be sequestered by the plasma membrane in human B-lymphoma cells (Sun *et al.*, 2005), suggesting that improper compartmentalization of Apaf-1 can also render tumor cells resistant to apoptotic stimuli. The heat shock proteins (HSPs) HSP27 and HSP70 can also prevent pro-caspase-9 activation at the apoptosome level. HSP27 binds to cytochrome *c* and prevents cytochrome *c*-mediated interaction of Apaf-1 with pro-caspase-9 (Bruey *et al.*, 2000), whereas HSP70 binds to Apaf-1 and prevents Apaf-1 oligomerization, thereby preventing the activation of pro-caspase-9 (Beere *et al.*, 2000). Thus, HSPs might be involved in resistance to anticancer therapy.

In neuroblastomas, the gene for caspase-8 is frequently inactivated by gene deletion or methylation. Caspase-8-deficient neuroblastoma cells are resistant to apoptosis

induction by doxorubicin (Teitz *et al.*, 2000). Similarly, inactivating mutations of caspase-8 gene are also observed in gastric carcinomas (Soung *et al.*, 2005).

Moreover, death receptors are downregulated or inactivated in many tumors. The expression of the death receptor CD95 is reduced in some tumor cells including hepatocellular carcinomas, neoplastic colon epithelium, and melanomas (Strand *et al.*, 1996). Several CD95 gene mutations have been reported in primary samples of myeloma (Landowski *et al.*, 1997). Mutation, inactivation or loss of CD95, might interfere with the induction of apoptosis in these types of cancer. Deletions and mutations of other death receptors, such as TRAIL-R1 and TRAIL-R2, have also been observed in metastatic breast cancer (Shin *et al.*, 2001).

1.3.2.3 Other mechanisms

As p53 is a well-known tumor suppressor that has a central function in apoptosis induction, alterations of the p53 pathway could influence the sensitivity of tumors to apoptosis. Studies in tissue culture have indicated that p53 deletion can render fibroblasts (Lowe *et al.*, 1993) and colon cancer cells (Bunz *et al.*, 1999) resistant to the induction of apoptosis by various anticancer drugs *in vitro*. p53-deficient mice and transgenic mice that express mutant p53 showed a poor response to apoptotic stimuli (Lee and Bernstein, 1993). Specific mutations of p53 have been linked to primary resistance to doxorubicin

treatment and early relapse in patients with breast cancer (Aas *et al.*, 1996). Moreover, the p53-related protein, p73, mediates resistance to cisplatin in epidermoid carcinoma cells by blocking p53 activity (Hayashi *et al.*, 2006).

The transcription factor nuclear factor kappa B (NF-κB) plays an important role in apoptosis. In viable cells, NF-κB is retained in the cytoplasm by binding to specific inhibitor called inhibitor of NF-κB (IκB). Apoptotic stimuli, however, can lead to phosphorylation, ubiquitination, and the subsequent degradation of IκB. Free NF-κB translocates to the nucleus where it activates the expression of several anti-apoptotic genes including IAPs and Bcl-x_L (Karin, 2006). Cells that express constitutively activated NF-κB are resistant to various chemotherapeutic agents (Paillard, 1999), and enhanced NF-κB activity has been linked to increased expression of the anti-apoptotic genes (Karin, 2006). Constitutive NF-κB activity confers resistance to gemcitabine in pancreatic carcinoma (Budihardjo *et al.*, 1999) and to paclitaxel in prostate cancer (Flynn *et al.*, 2003). Conversely, NF-κB inhibition sensitizes retinoblastoma cells to doxorubicin (Poulaki *et al.*, 2002).

1.4 Bcl-2 interacting mediator of cell death

Bim (Bcl-2 interacting mediator of cell death) is a BH3-only Bcl-2 family protein. It

was originally identified by screening a bacteriophage λ cDNA expression library, using the ^{32}P-labeled recombinant Bcl-2 protein as the probe (O'Connor *et al.*, 1998). By using yeast-two hybrid system, Bim was independently identified as a death gene in ovarian tissues (Hsu *et al.*, 1998). Bim has three major isoforms (Bim_{EL}, Bim_L, and Bim_S) which are generated by alternative splicing. Bim_{EL} is the largest isoform and is encoded by exons 2, 3, 4, 5 and 6 of the Bim gene. Bim_L is identical to Bim_{EL} but lacks exon 3. Bim_S is the shortest isoform that includes only exon 2, 5 and 6 (Bouillet *et al.*, 2001). All three isoforms have a hydrophobic C-terminus which is essential for their insertion into the membrane structures in the cytosol and a BH3 domain which is essential for their pro-apoptotic activity (O'Connor *et al.*, 1998). The three isoforms of Bim have different apoptotic potencies, with Bim_S being the most effective killer. This can be explained by the existence of additional regions in Bim_{EL} and Bim_L that attenuate their pro-apoptotic activity. Coimmunoprecipitation experiments demonstrated that Bim_{EL} and Bim_L, but not Bim_S, bind and sequester to LC8 of the microtubule-associated dynein motor complex, thereby blocking their pro-apoptotic activity under normal conditions (Puthalakath *et al.*, 1999). For exertion of pro-apoptotic functions, Bim binds to and antagonizes the anti-apoptotic Bcl-2 family proteins including Bcl-2, Bcl-x_L and Mcl-1, or directly binds to and activates the pro-apoptotic Bax-like proteins (Marani *et al.*, 2002). Modulating the activities of Bcl-2 family proteins by Bim ultimately leads to the initiation of intrinsic

death pathway. Bim is found in hematopoietic, epithelial, neuronal and germ cells, with Bim_{EL} being the predominant isoform. In contrast, Bim_S is less frequently detected in these tissues (O'Reilly *et al.*, 2000).

1.4.1 Role of Bim in apoptosis

The involvement of Bim in apoptosis was first implicated in a physiological study using gene-knockout mice (Bouillet *et al.*, 1999). Bim-deficient mice have an elevated level of lymphoid and myeloid cells. Moreover, $Bim^{-/-}$ lymphocytes were resistant to apoptotic stumuli such as cytokine deprivation, calcium ion flux, and microtubule perturbation. In a later study, Bim was found essential to the elimination of autoreactive thymocytes in the immune system (Bouillet *et al.*, 2002), since thymocytes lacking Bim were found to be resistant to the apoptosis induced by T-cell receptor stimulation. Besides the cells in the hematological system, Bim is also required for apoptosis in neurons (Putcha *et al.*, 2001) and osteoclasts (Akiyama *et al.*, 2003). In neurons, Bim deletion protected neurons from the apoptosis induced by nerve growth factor withdrawal (Putcha *et al.*, 2001). In osteoclasts, Bim deficiency conferred resistance to the cytokine withdrawal-induced apoptosis (Akiyama *et al.*, 2003).

Recent studies have highlighted the roles of Bim in tumorigenesis and response to a variety of chemotherapeutic drugs. Loss of a single allele of Bim induced the

development of mouse B cell leukemia in Myc-transgenic mice (Egle *et al.*, 2004). By screening with genome-wide array, homozygous deletion of Bim was found in the patients with mantle cell lymphoma (Tagawa *et al.*, 2005). In the context of chronic myeloid leukemia, it was found that Bcr-Abl-positive cell line K562 expresses low levels of Bim (Kuribara *et al.*, 2004). This phenomenon was recently confirmed in the primary leukemic cells from patients with chronic myeloid leukemia (Aichberger *et al.*, 2005). For the response of tumor cells to chemotherapeutic drugs, induction of Bim is required for the apoptosis induced by inhibitors of histone deacetylase in Saos-2 cells (Zhao *et al.*, 2005). Silencing of Bim expression in these cells resulted in complete abrogation of the histone deacetylase inhibitor-induced apoptosis. Likewise, Bim was required for paclitaxel responsiveness *in vitro* and in tumors *in vivo* (Tan *et al.*, 2005). Recently, Bim was found essential to the glucocorticoids-induced apoptosis in T-ALL cells (Lu *et al.*, 2006). Downregulation of Bim by shRNA significantly inhibited the glucocorticoids-induced apoptosis (Lu *et al.*, 2006). Apparently, Bim has a very important role in anticancer therapy.

1.4.2 Regulation of Bim

The expression and pro-apoptotic activity of Bim are subjected to complex modes of regulation. Both transcriptional and post-transcriptional mechanisms have been

documented.

1.4.2.1 Transcriptional regulation of Bim

Several pathways have been identified in the transcriptional regulation of Bim expression (Puthalakath and Strasser, 2002). The mode of regulation is depended on cell type and the nature of stimulus. In neurons and hematopoietic cells, Bim expression was elevated after growth factor withdrawal (Dijkers *et al.*, 2000; Putcha *et al.*, 2001). The growth factor withdrawal-induced Bim expression requires activation of the c-Jun N-terminal kinase (JNK) pathway in neurons (Dijkers *et al.*, 2000), but requires activation of the Akt pathway in hematopoietic cells (Putcha *et al.*, 2001). In fibroblasts and breast epithelial cells, Bim expression is depended on the inactivation of the extracellular signal-regulated kinase (ERK) pathway (Reginato *et al.*, 2003; Weston *et al.*, 2003).

1.4.2.2 Post-transcriptional regulation of Bim

In addition to the transcriptional regulation, the expression and pro-apoptotic activity of Bim can also be regulated by post-transcriptional mechanism. To date, the only identified mechanism by which Bim can be regulated post-transcriptionally is phosphorylation. ERK is the first kinase shown to be responsible for Bim phosphorylation. *In vitro* kinase experiments have revealed that ERK can phosphorylate recombinant

Bim_{EL}, but not Bim_L and Bim_S, at Ser-69. Moreover, transiently expressed Bim_{EL} in fibroblasts can be phosphorylated by the serum-activated ERK (Luciano *et al.*, 2003; Ley *et al.*, 2004). One of the biological consequences of ERK phosphorylation is the targeting of Bim_{EL} for ubiquitination and degradation by the proteosome. Initial evidence of ERK-dependent degradation of Bim_{EL} was obtained from fibroblasts (Ley *et al.*, 2003), and was subsequently found in lymphocytes (Mouhamad *et al.*, 2004) and mouse sympathetic neurons (Lang-Rollin *et al.*, 2004). Mutation at Ser-69 of Bim_{EL} completely blocked its turnover (Ley *et al.*, 2004), indicating that ERK phosphorylation of Bim_{EL} at this residue is a critical signal for proteosomal degradation. Another biological effect of ERK phosphorylation of Bim_{EL} is the prevention of its interaction with Bax. Phosphorylation of Bim_{EL} at Ser-59, Ser-69 or Ser-104 significantly reduced Bax-Bim_{EL} interaction, thereby preventing Bax oligomerization and apoptosis (Harada *et al.*, 2004).

Another kinase responsible for Bim phosphorylation is JNK. Studies in fibroblasts revealed that JNK could phosphorylate the recombinant Bim_L *in vitro* at Thr-56, and enforced expression of activated JNK caused the appearance of phosphorylated forms of Bim_L *in vivo* (Lei and Davis, 2003). JNK phosphorylation of Bim_L caused its release from sequestration by the dynein motor complex, and is thus able to trigger apoptosis (Lei and Davis, 2003). JNK can also phosphorylate Bim_{EL}. In neurons, JNK was found to phosphorylate Bim_{EL} at Ser-69 in response to nerve growth factor withdrawal (Putcha *et*

al., 2003). The initial evidence has shown that phosphorylation of Bim_{EL} by JNK potentiates apoptosis, but the detailed mechanisms still remain uncertain.

Recently, two other kinases have been shown capable to phosphorylate Bim. *In vitro* kinase assays showed that Akt could phosphorylate recombinant Bim_{EL}, and the phosphorylation site was identified as Ser-87 (Qi *et al.*, 2006). Phosphorylation by Akt *in vivo* led to the proteosomal degradation of Bim_{EL} (Qi *et al.*, 2006). p38-mediated phosphorylation of Bim_{EL} at Ser-65 (equivalent to Ser-69 of human Bim_{EL}) was demonstrated in rat PC-12 cells (Cai *et al.*, 2006). In contrast to Akt, p38 phosphorylation of Bim_{EL} was linked to the induction of apoptosis (Cai *et al.*, 2006).

1.5 c-Jun N-terminal kinase

The c-Jun N-terminal kinase (JNK) is a member of the mitogen-activated protein kinase (MAPK) superfamily and has been demonstrated to play a critical role in apoptosis (Davis, 2000). JNK has three isoforms, namely JNK1, JNK2, and JNK3. JNK1 and JNK2 are widely expressed in most tissues. In contrast, the expression of JNK3 is largely limited to the brain, heart, and testis (Davis, 2000). The role of JNK in apoptosis is still under debate, as JNK has been reported to have both pro-apoptotic and anti-apoptotic functions, depending largely on the cell type and nature of stimulus (Lin, 2003).

1.5.1 Pro-apoptotic role of JNK

There is numerous evidence showing that JNK functions as a pro-apoptotic kinase. Initial studies had been performed on neuronal cells. In rat PC-12 cells, nerve growth factor-induced apoptosis was efficiently blocked by expressing a dominant-interfering form of JNK. In contrast, activation of the JNK pathway in these cells by expressing a constitutively active JNK induced apoptosis (Xia *et al.*, 1995). The pro-apoptotic role of JNK was further demonstrated in several gene disruption studies. Hippocampal neurons of mice with disrupted JNK3 gene were defective in the apoptotic response to excitotoxin (Yang *et al.*, 1997). Moreover, reduced apoptosis was found in the hindbrain neuroepithelium of mice lacking both JNK1 and JNK2 (Sabapathy *et al.*, 1999). JNK is also required for apoptosis in non-neuronal cells. Primary murine embryo fibroblasts lacking that lack the expression of both JNK1 and JNK2 exhibited profound defect in apoptosis induced by UV radiation, anisomysin and methylmethanesulfonate (Tournier *et al.*, 2000).

1.5.2 Anti-apoptotic role of JNK

In comparison, the anti-apoptotic function of JNK is not so well-characterized, but it has also been implicated in some earlier studies. Antisense oligonucleotides of JNK inhibited the growth of lung carcinoma cells (Bost *et al.*, 1999). In addition, enhanced

skin papilloma development induced by 12-O-tetradecanoylphorbol-13-acetate was observed in JNK1 null mice (She *et al.*, 2002). Recently, the JNK-mediated cell survival had been demonstrated in lymphocytes, and the underlying mechanism was resolved (Yu *et al.*, 2004). In an IL-3 dependent cell line, IL-3 withdrawal-induced apoptosis was promoted by JNK inhibition but suppressed by overexpressing a constitutively active JNK. Furthermore, JNK was found to exert its anti-apoptotic function by phosphorylating Bad, thus reducing the Bad/Bcl-x_L interaction (Yu *et al.*, 2004).

1.6 Hypotheses

Approximately 20% of the patients with T-ALL are not responsive to the current chemotherapeutic regimens, and these patients will eventually die of the disease. Unfortunately, the cause of treatment failure in these patients remains largely unknown. As chemotherapy acts primarily by inducing apoptosis, defects in the apoptotic pathway may also contribute to the chemoresistance in at least some T-ALL patients. Using cell-based models, we aim to investigate the molecular mechanisms that render T-ALL cells resistant to drug-induced apoptosis. Findings of this study would be expected to facilitate the development of novel treatment regimens or strategies against drug-resistant T-ALL.

Chapter 2 Materials and Methods

2.1 Cell culture

The human T-ALL cell lines, HSB-2, MOLT-3 and Sup-T1, as well as the Burkitt lymphoma-derived cell line, Raji, were obtained from the American Type Culture Collection (Rockville, MD). HSB-2 was derived from a buffy coat preparation from a patient with ALL (Adams *et al.*, 1968). MOLT-3 was established from a T-ALL patient in relapse (Minowada *et al.*, 1972). Sup-T1 was derived from malignant cells collected from pleural effusion of an 8-year-old child with T-ALL (Smith *et al.*, 1984). Raji was established from a Burkitt lymphoma of the left maxilla of an 11-year-old male (Pulvertaft, 1964). All cell lines were grown in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Rockville, MD), 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES (*N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid), 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were maintained in 5% CO_2 at 37 °C. Where indicated, Sup-T1 cells were incubated with different inhibitors including MG-132 (50 μM), U0126 (10 μM), PD98509 (10 μM), SP600125 (10 μM), SB203580 (15 μM), LY294002 (50 μM), or wortmannin (100 nM), for the specified time points. All inhibitors were obtained from Calbiochem, La Jolla, CA.

2.2 Induction and quantification of apoptosis

To induce apoptosis, the leukemic cells were seeded at an initial density of 1.5×10^5 cells/mL and treated with or without 1 μM, 5 μM or 10 μM etoposide (Sigma) for the indicated time points. In some experiments, the cells were preincubated with 50 μM z-VAD-fmk (N-benzyloxycarbonyl-Val-Ala-aspartyl-fluoromethyl ketone; Calbiochem) for 1 hour prior to the etoposide treatment. After treatment, the cells were washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol at -20 °C for 24 h. The cells were next incubated in dark for 30 min with 1 mg/mL ribonuclease A (RNase A; Sigma) and 10 μg/mL propidium iodide (PI; Sigma). Samples were analyzed for DNA content by measuring PI fluorescence on the Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL). Cells displaying hypodiploid DNA content were quantified and regarded as the apoptotic population.

2.3 Determination of caspase activities

Caspase-3 and caspase-9 activities were determined by using the fluorogenic peptide substrates, DEVD-AFC (50 μM), and LEHD-AFC (100 μM) (Calbiochem), respectively. Cell lysates from 1×10^6 cells and substrates were mixed in a standard reaction buffer (20 mM HEPES, 10% glycerol, 2 mM dithiothreitol (DTT), pH 7.5), and incubated for 1 hour at 37 °C. The amount of enzyme-catalyzed AFC release was measured by a fluorescence

plate reader (Tecan, Grödig, Austria) with an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Fluorescence values were converted into picomoles of AFC release by using a standard curve generated with free AFC.

2.4 Western blotting

2.4.1 Protein extraction and determination of protein concentration

For preparation of whole cell lysates, cells were harvested and suspended for 30 minutes on ice in an extraction buffer (20 mM Tris-HCl at pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.2% BSA, 1% Triton X-100) supplemented with protease inhibitors (BD Biosciences, San Jose, CA). Lysates were clarified by centrifugation at 4 °C for 15 minutes at 15 000*g*. The resulting supernatants were frozen at -20 °C or used immediately. Aliquots of the supernatants were used for protein concentration determination by the standard bicinchoninic acid assay kit (Pierce, Rockford, IL).

To obtain mitochondrial and cytosolic fractions, homogenates from leukemic cells were fractionated by the Cytosol-Mitochondria Fractionation kit (Calbiochem). Briefly, 1 × 10^7 cells were harvested and resuspended in cytosol extraction buffer supplemented with protease inhibitors for 10 minutes on ice, and disrupted using a Dounce homogenizer. Homogenates were then centrifuged at 700*g* at 4 °C for 10 minutes. The supernatants were further centrifuged at 10 000*g* for 30 minutes at 4 °C. The resulting supernatants

(cytosolic fraction) and pellets (mitochondrial fraction) were recovered.

2.4.2 SDS-PAGE and immunodetection

Samples (30 – 100 μg protein) were denatured in Laemmli buffer and resolved on 10 or 13% sodium docecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) minigels, followed by electrophoretic transfer to nitrocellulose membranes (Hybond ECL; Amersham, Buckinghamshire, UK). The membranes were blocked for 1 h in Tris-buffered saline with 0.1% Tween-20 containing 5% non-fat dry milk. Immunodetection was performed by overnight incubation at 4 °C with primary antibodies diluted in the same blocking buffer. Primary antibodies to PARP, caspase-3, caspase-9, Bcl-x_L, Bak, Bad, Bim, Bid, phospho-ERK (Thr-202/Tyr-204), ERK, phospho-JNK (Thr-183/Tyr-185), JNK, phospho-p38 (Thr-180/Tyr-182), p38, phospho-Akt (Ser-473), Akt, phospho-c-Jun (Ser-63), c-Jun and cytochrome *c* were obtained from Cell Signalling Technology, Beverly, MA. Antibodies to Bcl-2, Mcl-1, Bax, Bik and Nip-3 were purchased from BD Biosciences, while antibody to β-actin was obtained from Sigma. After extensive washing, the blots were probed with horseradish peroxidase-conjugated secondary antibodies (Cell Signalling Technology) for 1 hour at room temperature, and proteins were visualized by enhanced chemiluminescence (Cell Signalling Technology). Primary antibodies were employed at a 1:250 to 1:5000 dilution, and secondary

antibodies were employed at a 1:2000 dilution.

2.5 Cell-free apoptosis reactions

For preparation of S-100 cytosolic extracts, 5×10^7 cells were collected and incubated for 10 minutes in ice-cold S-100 buffer (20 mM HEPES-KOH at pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) supplemented with protease inhibitors. Cells were disrupted using a Dounce homogenizer, and centrifuged at 1000*g* for 10 minutes at 4 °C. Supernatants were further centrifuged at 100 000*g* for 1 hour, and the resulting supernatants (S-100 fraction) were stored at -70 °C or used immediately. For cell-free caspase activation, S-100 cytosol (100 μg) was incubated with 1 mM dATP (deoxyadenosine triphosphate) and 4 μM cytochrome *c* (Sigma) for 1 hour at 30 °C. Aliquots of reaction products were then collected for assessment of pro-caspase-3 processing by Western blotting.

2.6 Analysis of mitochondrial membrane potential

Loss of mitochondrial membrane potential ($\Delta\Psi_m$) was detected by using the potential-sensitive fluorescent probe, tetramethylrhodamine ethyl ester (TMRE; Molecular Probes, Eugene, OR). Treated or untreated cells were incubated with 25 nM TMRE for 15 minutes at 37 °C prior to analysis on a flow cytometer. As a positive control,

cells were incubated with 100 μM potential-disrupting agent, carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma) for 24 hours. Data are displayed as the percentage of cells with reduction in mitochondrial membrane potential.

2.7 Transient transfection of Sup-T1 cells

The Bcl-2 antisense oligonucleotide, that targets the first 6 codons of the opening reading frame of Bcl-2 mRNA, was purchased from Calbiochem. The full-length, wild type human Bim_{EL} and Bax cDNA, which are cloned into the expression vector pCMV6-XL5, were obtained from Origene, Rockville, MD. Transient transfection of Sup-T1 cells was accomplished using Lipofectin reagent (Invitrogen, Carlsbad, CA) under serum-free conditions. Briefly, 2.5×10^6 cells were transfected with either 3 μg of Bcl-2 antisense oligonucleotide or 5 μg of Bim_{EL} or Bax expression vector. After 24 hours of transfection, the medium was replaced with complete medium, and the cells were treated with or without etoposide for 24 hours. The cells were then harvested for analysis of gene expression, and processing of pro-caspase-3 and PARP by Western blotting.

2.8 Reverse transcription-polymerase chain reaction (RT-PCR)

2.8.1 RNA isolation

Total RNA was isolated from leukemic cells by using TRIzol reagent (Invitrogen).

Briefly, the cells were pelleted by centrifugation, and lysed in TRIzol reagent for 5 minutes. Chloroform (0.2 mL/mL TRIzol reagent) was then added to the homogenates, and the samples were mixed vigorously for 15 seconds. Subsequently, the mixture was centrifuged at 12 000*g* for 10 minutes at 4 °C. After centrifugation, RNA in the aqueous phase was precipitated by mixing with isopropyl alcohol (0.5 mL/mL TRIzol reagent). The RNA pellet was washed with 75% ethanol, and dissolved in RNase-free water.

2.8.2 Synthesis of first-strand cDNA

First-strand cDNA was synthesized by reverse transcription using the SuperScript First Strand Synthesis System (Invitrogen). Briefly, 2 μg of DNase I-treated RNA was reversely transcribed for 1 hour at 42 °C in a reaction mix containing 1× reverse transcription buffer, 50 ng random hexamers, 0.5 mM dNTPs, 5 mM $MgCl_2$, 10 mM DTT, 20 U RNaseOUT RNase inhibitor and 50 U SuperScript II reverse transcriptase. The reaction was terminated at 70 °C for 15 minutes. Subsequently, 1 U RNase H was added to the samples to remove the RNA template. The cDNA was stored at -20 °C or used immediately for PCR.

2.8.3 Polymerase Chain Reaction

All PCR reagents were purchased from Amersham. The first-strand cDNA was

diluted and used as template in PCR reactions containing 0.8 μM primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer and 0.5 U Taq polymerase. The PCR reaction was subjected to 3 min denaturation at 96 °C, followed by 25 cycles of amplification (94 °C, 30 s; 55 °C, 30 s; 72 °C, 60 s) and a final extension at 72 °C for 10 min. The gene-specific primer pairs were: Bax-sense (5'-CTG AGC AGA TCA TGA AGA CAG G-3') and Bax-antisense (5'-AAG TAG AAA AGG GCG ACA ACC-3'); Bcl-2-sense (5'-GAA TTC CAC TGT CAA GAA AGA GCA GT-3') and Bcl-2-antisense (5'-GCT TTC GAA ATA TCA ACC ACA GCA TT-3'); Bim-sense (5'-ATG GCA AAG CAA CCT TCT GA-3') and Bim-antisense (5'-CGC ATA TCT GCA GGT TCA GCC-3'); GADPH-sense (5'-AAG ATC ATC AGC AAT GCC TCC-3') and GADPH-antisense (5'-CCT GCT TCA CCA CCT TCT TGA-3'). PCR products were separated by electrophoresis in 1% agarose gels, and were visualized by ethidium bromide staining.

2.9 Alkaline phosphatase digestion of Bim

Cell lysates (50 μg) were digested with 50 U of calf intestinal alkaline phosphatase (CIAP; Calbiochem) for 1 hour at 37 °C in a digestion buffer (50 mM Tris-HCl at pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) prior to Western blotting.

Chapter 3 Results

3.1 The T-ALL cell line Sup-T1 is resistant to etoposide-induced apoptosis

We first tested the response of a panel of established T-ALL cell lines, including HSB-2, MOLT-3 and Sup-T1, to the commonly used anticancer drug, etoposide. Raji, a Burkitt lymphoma-derived cell line, which was previously shown to be resistant to etoposide (Sun *et al.*, 2005) as well as to other chemotherapeutic agents (Kawabata *et al.*, 1999), was included as a control cell line.

Leukemic cells treated with 1 μM of etoposide for 24 hours were subjected to flow cytometric evaluation of DNA content using propidium iodide staining. Despite the cell cycles had been deregulated, less than 10% apoptosis (hypodiploid population) was detected in Raji and Sup-T1 cells after 24 hours of treatment. In contrast, more than 80% apoptosis was detected in HSB-2 and MOLT-3 cells (Fig 3.1 and 3.2).

To confirm these results, cell lysates from the leukemic cells were immunoblotted with specific antibody that recognizes full-length PARP and its cleaved form. Etoposide-triggered processing of intact PARP to its 89-kDa fragment was evident in MOLT-3 and HSB-2 cells but could not be detected in Raji and Sup-T1 cells, suggesting that etoposide is capable of inducing apoptosis in MOLT-3 and HSB-2 but not Raji and Sup-T1 cells (Fig. 3.3).

To ascertain whether apoptosis in Sup-T1 cells was merely delayed, we lengthened the incubation period to 48 hours. Results from flow cytometric analysis revealed that the response of the leukemic cells to etoposide was more or less the same upon an extended treatment period (Fig 3.4 and 3.5). Likewise, 5- and 10-fold increase in the dose of etoposide was still incapable of eliciting an apoptotic response in Sup-T1 cells. In Raji, although there was a dose-dependent increase of apoptosis (about 20% apoptosis when treated with 10 μM etoposide), resistance to etoposide was still prominent, when compared with the cases in MOLT-3 and HSB-2 cells (Fig 3.6 and 3.7).

Taken together, these data demonstrate that the T-ALL cell line Sup-T1 is completely resistant to etoposide-induced apoptosis. This prompted us to recruit Sup-T1 as a model cell line to unravel the possible resistance mechanisms of chemotherapy-induced apoptosis in T-ALL cells.

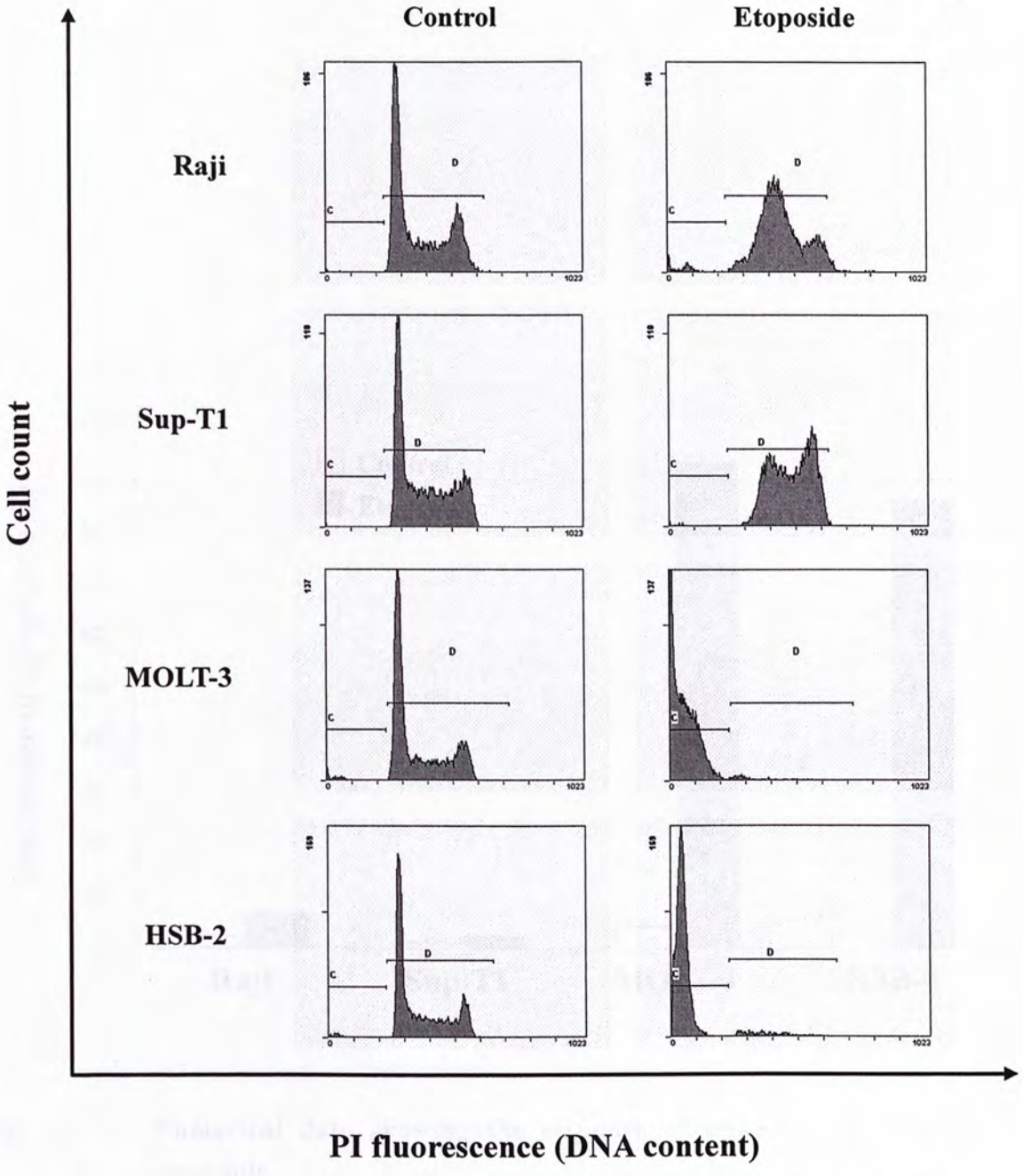

Fig. 3.1 **Representative DNA histograms showing the response of leukemic cell lines to etoposide.**

Leukemic cells were incubated for 24 hours in the absence (*control*) or presence of 1 μM etoposide, and subjected to flow cytometric analysis of DNA content using PI staining. Cells in gate C harbor hypodiploid DNA content, and they were defined as apoptotic cells; while cells in gate D harbor normal DNA content, and they were defined as non-apoptotic cells. Numerical data are shown in Fig. 3.2. The numbers in the x- and y-axes of the DNA histograms represent the relative fluorescence intensity and cell number, respectively.

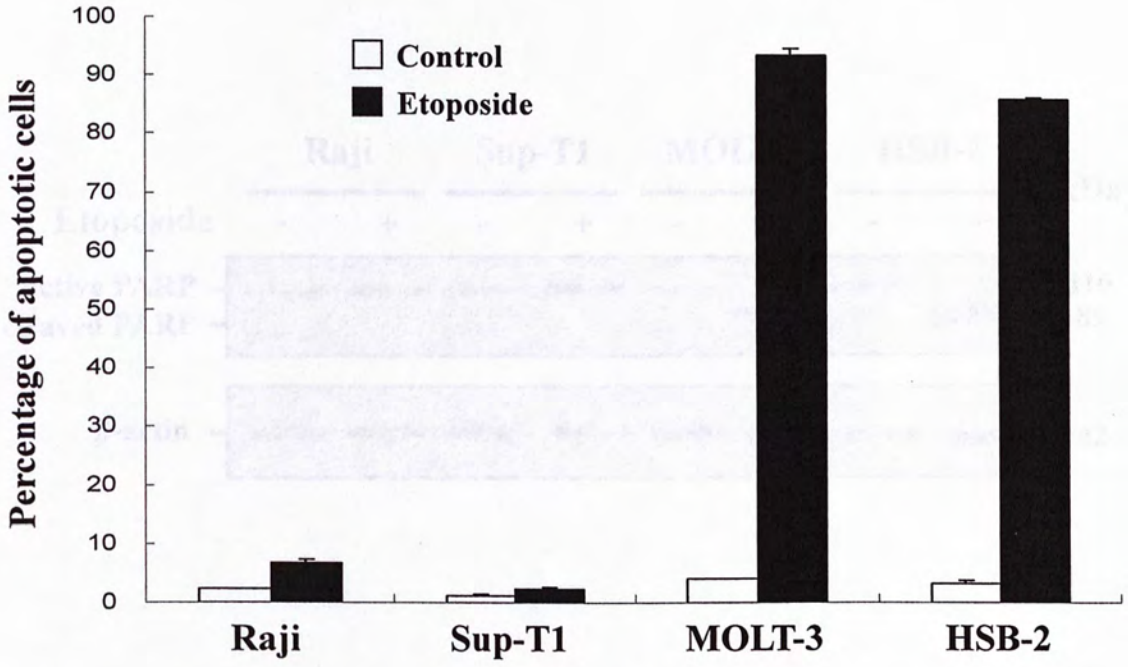

Fig. 3.2 **Numerical data showing the response of leukemic cell lines to etoposide.**

Leukemic cells were incubated for 24 hours in the absence (□) or presence (■) of 1 μM etoposide, and subjected to flow cytometric analysis of DNA content using PI staining. Cells with hypodiploid DNA content were counted, and their numbers are expressed as a percentage of total population. Results are shown as mean values ± S. D. (n = 3).

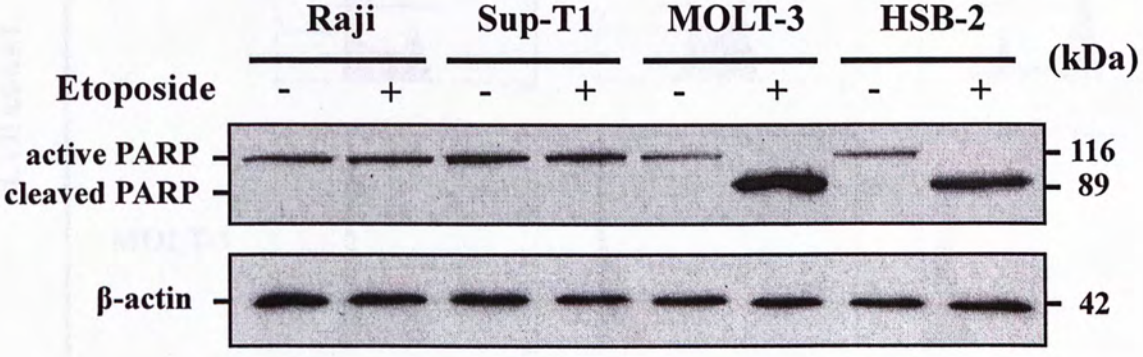

Fig. 3.3 **Representative immunoblot showing the PARP status of leukemic cells in response to etoposide.**

Lysates from leukemic cells treated for 24 hours with or without 1 μM etoposide were analyzed by Western blotting for the processing of PARP by using specific antibody. β-actin was used as a loading control.

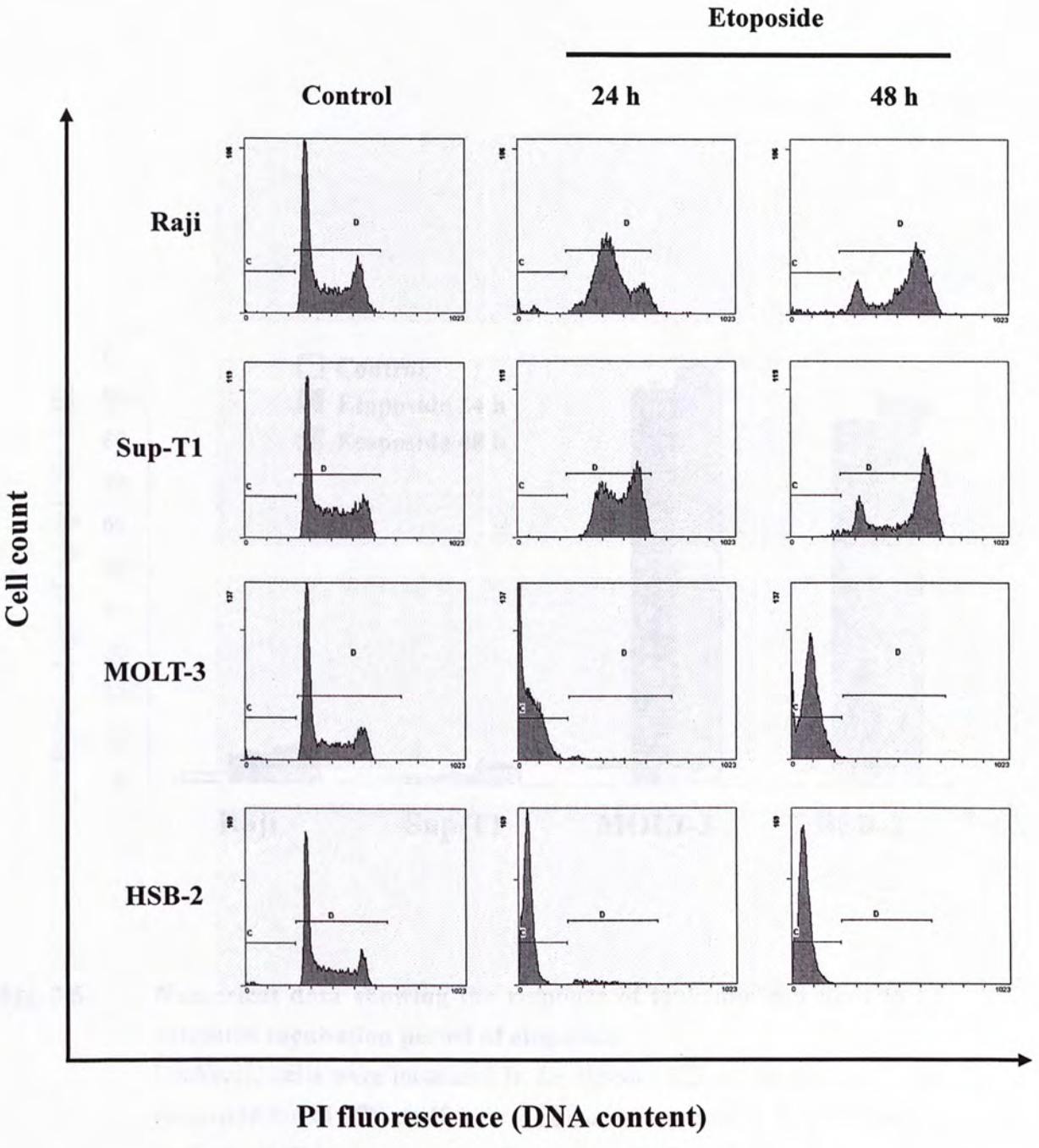

Fig. 3.4 **Representative DNA histograms showing the response of leukemic cell lines to an extended incubation period of etoposide.**

Leukemic cells were incubated in the absence (*control*) or presence of 1 μM etoposide for 24 or 48 hours, and subjected to flow cytometric analysis of DNA content using PI staining. Cells in gate C harbor hypodiploid DNA content, and they were defined as apoptotic cells; while cells in gate D harbor normal DNA content, and they were defined as non-apoptotic cells. Numerical data are shown in Fig. 3.5.

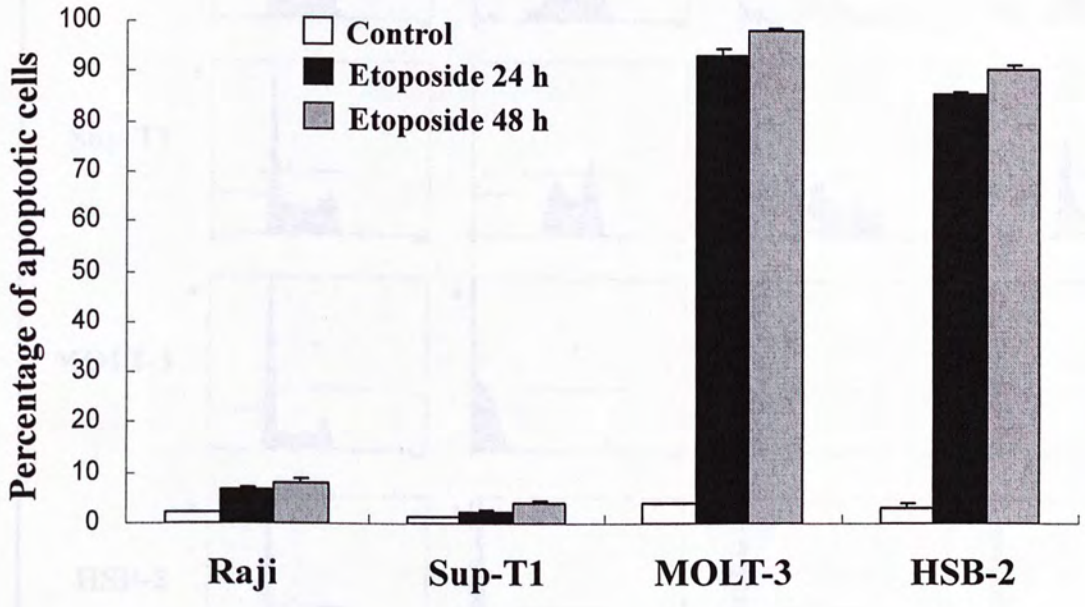

Fig. 3.5 **Numerical data showing the response of leukemic cell lines to an extended incubation period of etoposide.**

Leukemic cells were incubated in the absence (□) or presence of 1 μM etoposide for 24 (■) or 48 hours (▨), and subjected to flow cytometric analysis of DNA content using PI staining. Cells with hypodiploid DNA content were counted, and their numbers are expressed as a percentage of total population. Results are shown as mean values ± S. D. (n = 3).

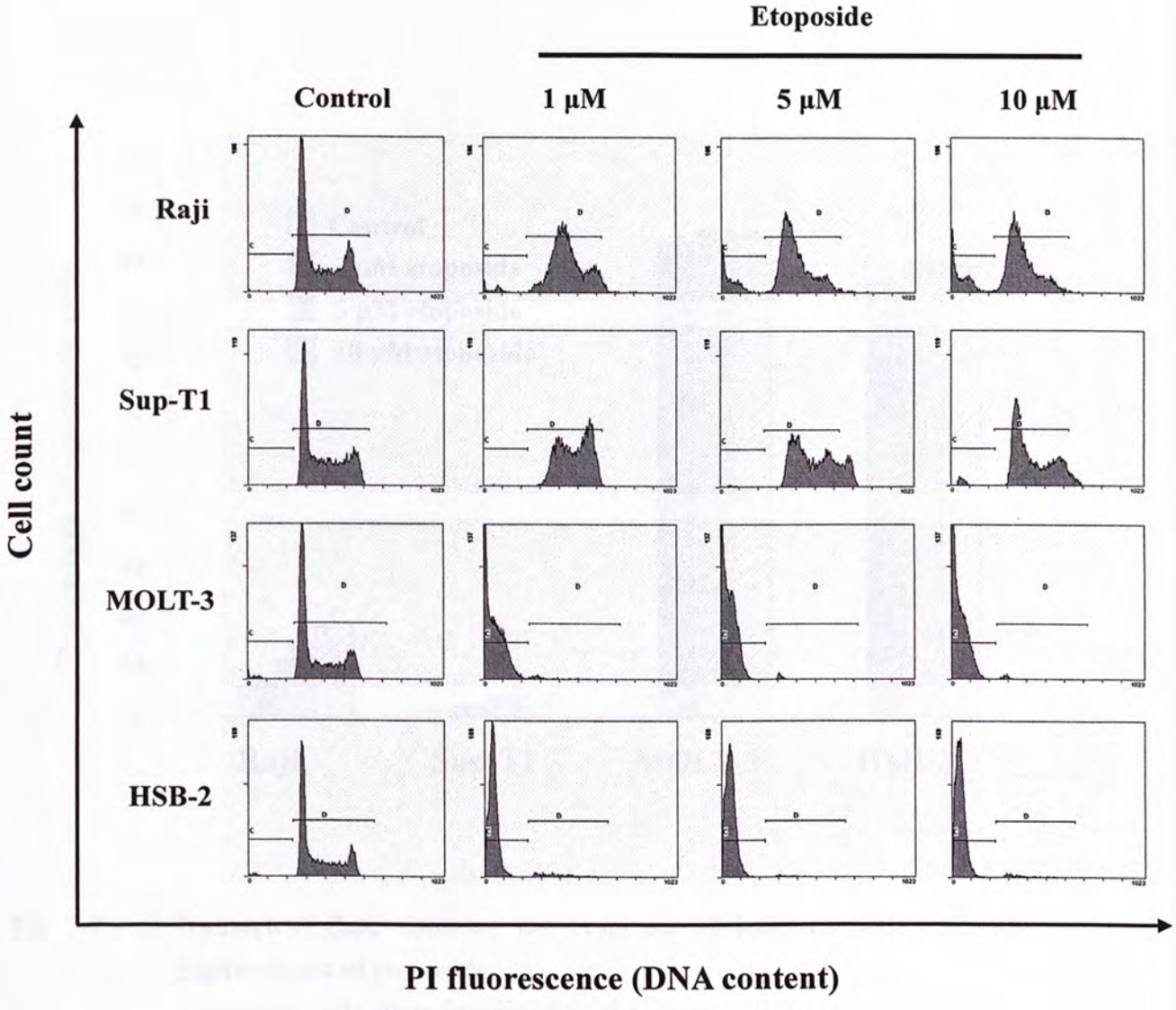

Fig. 3.6 **Representative DNA histograms showing the response of leukemic cell lines to higher doses of etoposide.**

Leukemic cells were incubated in the absence (*control*) or presence of 1, 5 or 10 μM etoposide for 24 hours, and subjected to flow cytometric analysis of DNA content using PI staining. Cells in gate C harbor hypodiploid DNA content, and they were defined as apoptotic cells; while cells in gate D harbor normal DNA content, and they were defined as non-apoptotic cells. Numerical data are shown in Fig. 3.7.

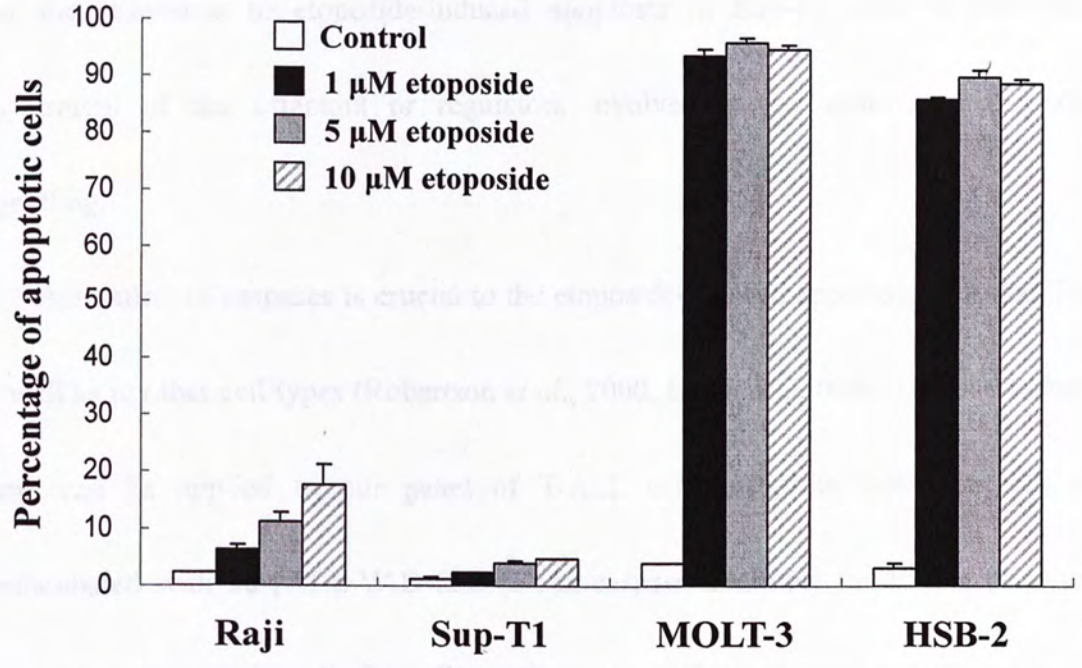

Fig. 3.7 **Numerical data showing the response of leukemic cell lines to higher doses of etoposide.**

Leukemic cells were incubated in the absence (□) or presence of 1 (■), 5 (▩) or 10 μM (▨) etoposide for 24 hours, and subjected to flow cytometric analysis of DNA content using PI staining. Cells with hypodiploid DNA content were counted, and their numbers are expressed as a percentage of total population. Results are shown as mean values ± S. D. (n = 3).

3.2 Sup-T1 cells are resistant to etoposide-induced caspase activation

Etoposide exerts its anticancer effect by inducing DNA damage and activating the mitochondrial-dependent apoptosis pathway (Robertson *et al.*, 2000). Thus, we believed that the resistance to etoposide-induced apoptosis in Sup-T1 cells is attributed to impairment of the effectors or regulators involved in the mitochondrial pathway signalling.

Activation of caspases is crucial to the etoposide-induced apoptosis in Jurkat T cells as well as in other cell types (Robertson *et al.*, 2000; Lin *et al.*, 2004). To test whether the same can be applied to our panel of T-ALL cell lines, the leukemic cells were preincubated with 50 μM z-VAD-fmk (a pan-caspase inhibitor) for 1 hour prior to the etoposide treatment. Results from flow cytometric analysis showed that apoptosis in the etoposide-sensitive MOLT-3 and HSB-2 cells was significantly blocked by preincubation with z-VAD-fmk, from more than 80% to less than 15% (Fig. 3.8 and 3.9), suggesting that caspases are playing a critical role in the etoposide-mediated apoptosis in the T-ALL cells. Therefore, the resistance to etoposide-induced apoptosis in Sup-T1 cells is likely due to defective caspase activation.

To investigate whether Sup-T1 cells are resistant to etoposide-induced caspase activation, cell lysates from the leukemic cells were immunoblotted with specific antibodies against caspase-3 and caspase-9. Etoposide-triggered processing of

pro-caspase-3 into the p17 active subunit was readily detected in MOLT-3 and HSB-2 cells (Fig. 3.10, *top panel*). Concomitant processing of pro-caspase-9 into its p35 and p37 active fragments was also evident in MOLT-3 and HSB-2 extracts (Fig. 3.10, *bottom panel*). However, processing of pro-caspase-3 and procaspase-9 were absent in the etoposide-treated Raji and Sup-T1 cells.

To verify these data, the cells treated with etoposide were subjected to measurement of caspase-3 and caspase-9 activities according to specific cleavage of the fluorescent substrates Ac-DEVD-AFC and Ac-LEHD-AFC, respectively. The results showed that neither Ac-DEVD-AFC (Fig. 3.11, *top panel*) nor Ac-LEHD-AFC cleavage (Fig. 3.11, *bottom panel*) could be detected in the etoposide-treated Raji and Sup-T1 cells; whereas, cleavage of these synthetic substrates were readily detected in HSB-2 and MOLT-3 cells upon the etoposide treatment. Taken together, these data demonstrate that Sup-T1 cells are resistant to the etoposide-induced caspase activation.

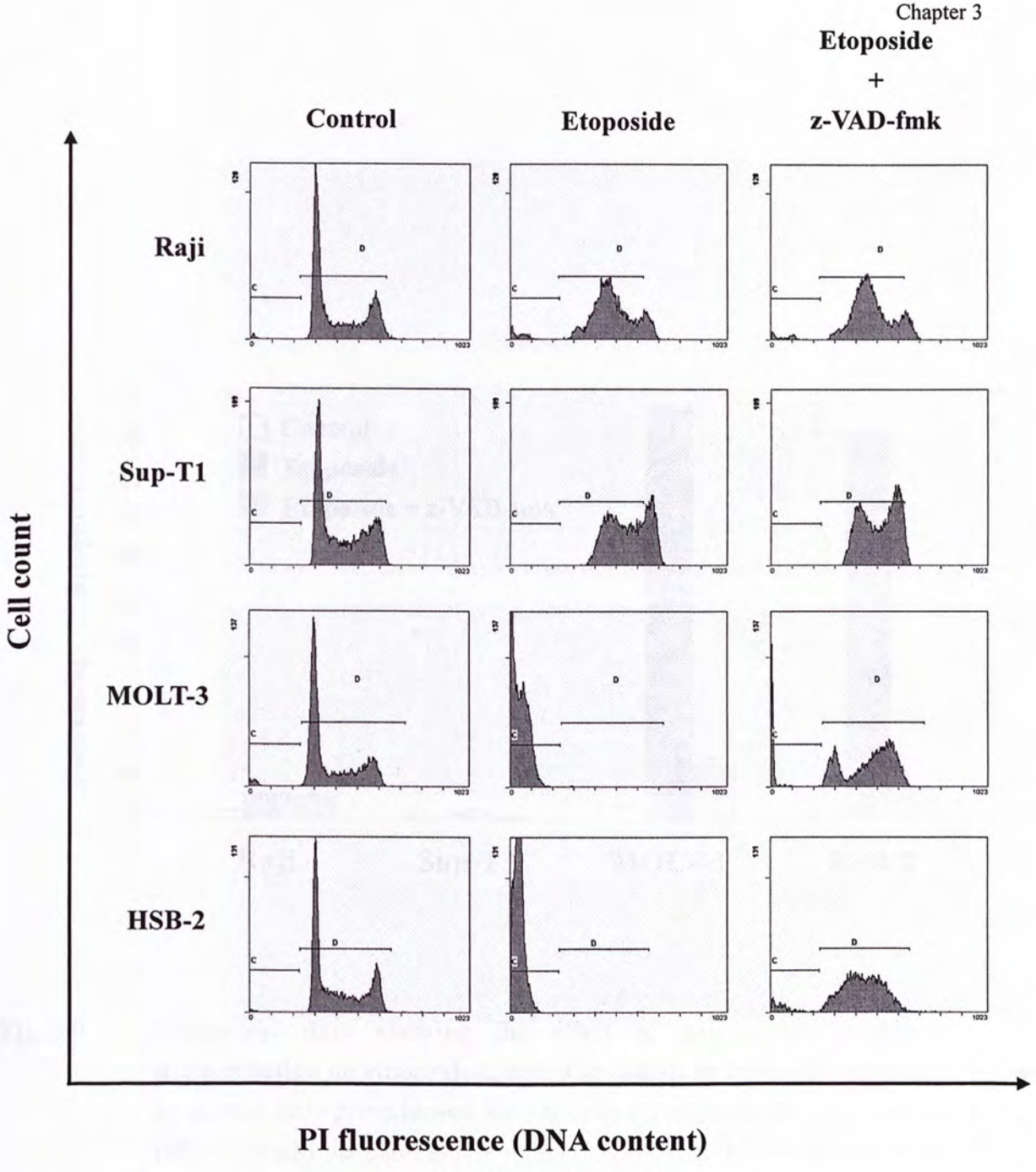

Fig. 3.8 **Representative DNA histograms showing the effect of pan-caspase inhibitor preincubation on etoposide-induced apoptosis in leukemic cell lines.**

Leukemic cells preincubated for 1 hour in the absence (*control*) or presence of 50 μM the pan-caspase inhibitor, z-VAD-fmk, were treated with 1 μM etoposide for 24 hours, and subjected to flow cytometric analysis of DNA content using PI staining. Cells in gate C harbor hypodiploid DNA content, and they were defined as apoptotic cells; while cells in gate D harbor normal DNA content, and they were defined as non-apoptotic cells. Numerical data are shown in Fig. 3.9.

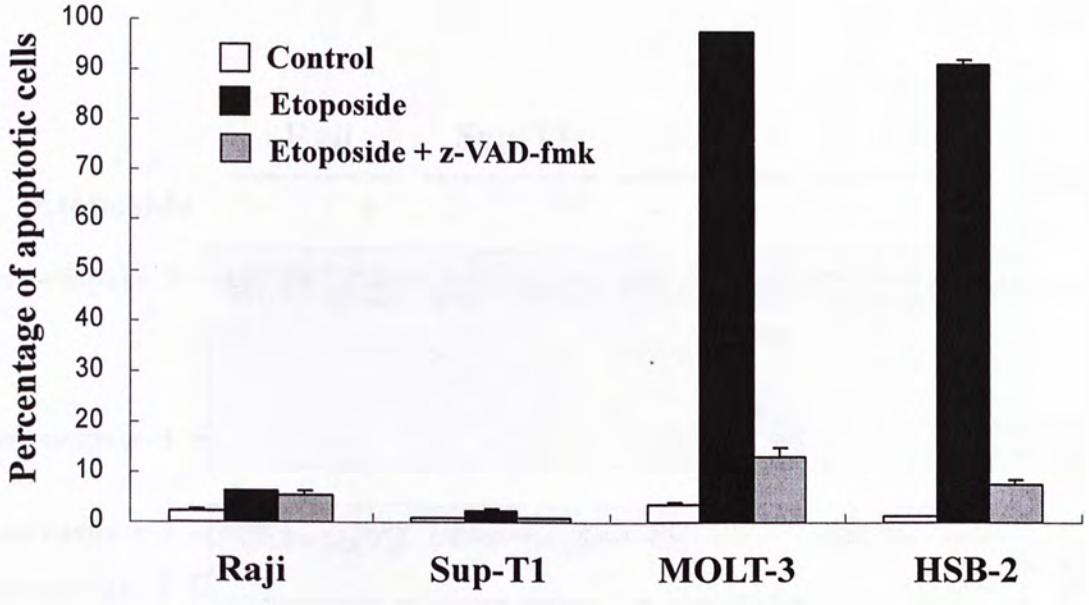

Fig. 3.9 **Numerical data showing the effect of pan-caspase inhibitor preincubation on etoposide-induced apoptosis in leukemic cell lines.** Leukemic cells preincubated for 1 hour in the absence (■) or presence (▒) of 50 μM the pan-caspase inhibitor, z-VAD-fmk, were treated with 1 μM etoposide for 24 hours, and subjected to flow cytometric analysis of DNA content using PI staining. (□) indicates control cells without pan-caspase inhibitor or etoposide treatment. Cells with hypodiploid DNA content were counted, and their numbers are expressed as a percentage of total population. Results are shown as mean values ± S. D. (n = 3).

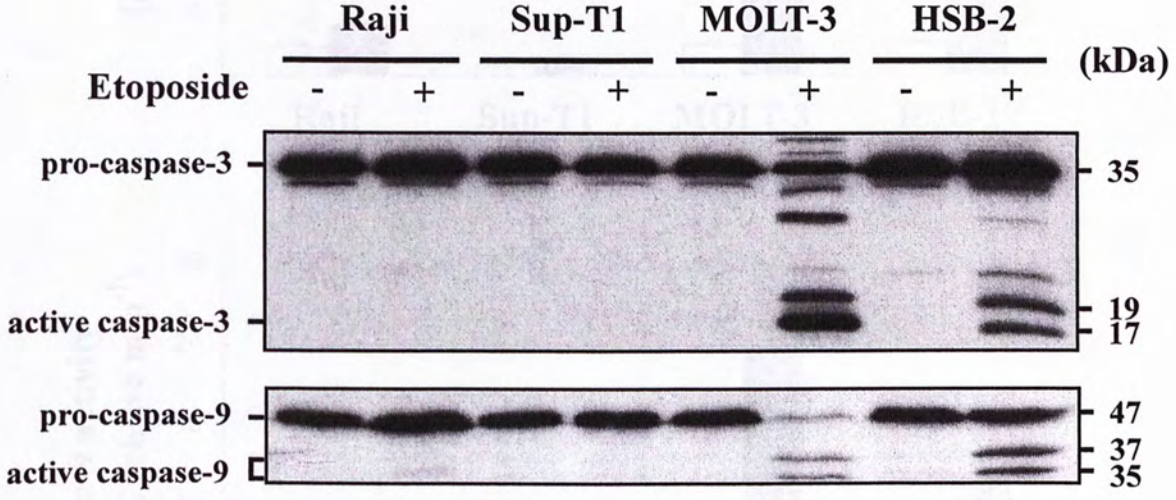

Fig. 3.10 **Representative immunoblots showing the status of caspase-3 and caspase-9 of leukemic cells in response to etoposide.**

Lysates from leukemic cells treated for 24 hours with or without 1 μM etoposide were analyzed by Western blotting for the processing of caspase-3 (*top panel*) and caspase-9 (*bottom panel*) by using specific antibodies.

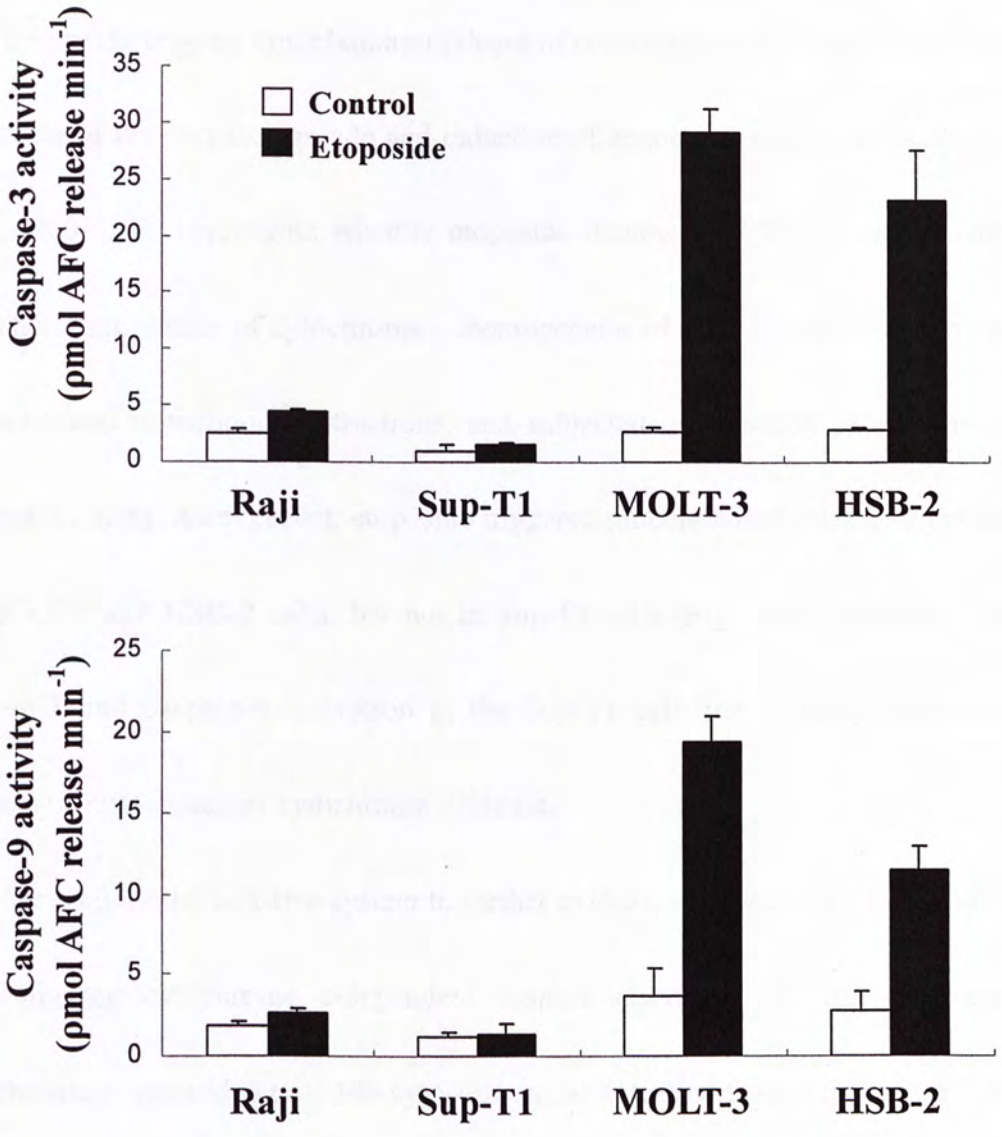

Fig. 3.11 **Graphs showing the effect of etoposide on caspase-3 and caspase-9 activity in leukemic cell lines.**

Cells treated for 24 hours with 1 μM etoposide were harvested for measurement of caspase-3 (*top panel*) and caspase-9 activity (*bottom panel*) according to specific cleavage of the fluorescent substrates DEVD-AFC and LEHD-AFC, respectively. Data are shown as mean values ± S. D. (n = 3), and depicted as pmol AFC release per minute.

3.3 Sup-T1 cells are insusceptible to etoposide-induced mitochondrial alterations

Etoposide triggers mitochondrial release of cytochrome *c* in cancer cells, resulting in activation of the caspase cascade and induction of apoptosis (Robertson *et al.*, 2000; Lin *et al.*, 2004). To investigate whether etoposide treatment of T-ALL cells could induce mitochondrial release of cytochrome *c*, homogenates of T-ALL cells were separated into cytosolic and mitochondrial fractions, and subjected to detection of cytochrome *c* by Western blotting. As expected, etoposide triggered mitochondrial release of cytochrome *c* in MOLT-3 and HSB-2 cells, but not in Sup-T1 cells (Fig. 3.12). Therefore, defective caspase-3 and caspase-9 activation in the Sup-T1 cell line is likely attributed to the absence of mitochondrial cytochrome *c* release.

We then used a cell-free system to further evaluate whether Sup-T1 cells are capable of promoting cytochrome *c*-dependent caspase activation. To this end, exogenous cytochrome *c* was added to S-100 cytosolic extracts derived from Raji and the T-ALL cell lines. Processing of pro-caspase-3 into the p17 active fragment was readily detected in the etoposide-sensitive HSB-2 and MOLT-3 cells, as well as in the etoposide-resistant Sup-T1 cells (Fig. 3.13). Thus, defective caspase activation in Sup-T1 cells is due to the absence of mitochondrial cytochrome *c* release. These data also indicate that the death signalling downstream of mitochondria in Sup-T1 cells is robust. In Raji cells, despite etoposide could induce mitochondrial release of cytochrome *c* (Fig. 3.12), results from cell-free

apoptosis reactions showed that addition of exogenous cytochrome *c* could not restore caspase activation (Fig. 3.13). These observations are consistent with those published in a recent report, which clearly showed that the resistance to etoposide-induced apoptosis in Raji cells is blocked downstream of cytochrome *c* release, yet upstream of caspase-3 activation (Sun *et al.*, 2005).

To further delineate the apoptosis defect in Sup-T1 cells, we tested whether etoposide could dissipate the mitochondrial membrane potential ($\Delta\Psi_m$) in T-ALL cell lines by flow cytometry using a potential-sensitive fluorescent probe, TMRE. Etoposide-triggered mitochondrial depolarization was evident in HSB-2 and MOLT-3 but not Sup-T1 cells (Fig. 3.14). Treatment with an uncoupling agent, CCCP, however, dissipated the mitochondrial membrane potential in Sup-T1 cells (Fig. 3.14). Altogether, these data imply that the defective caspase activation in Sup-T1 cell line is ascribed to its insusceptibility to the etoposide-induced mitochondrial alterations.

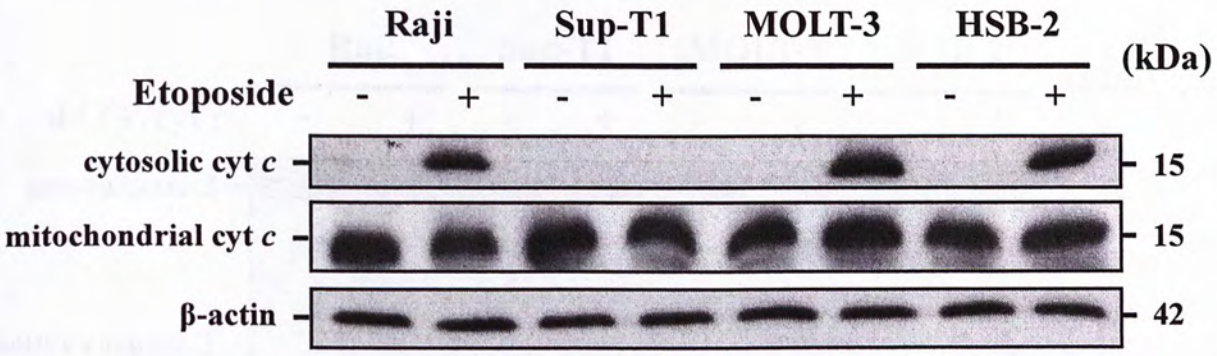

Fig. 3.12 **Representative immunoblots showing the effect of etoposide on the mitochondrial release of cytochrome *c* in leukemic cell lines.**
Homogenates from the leukemic cells treated for 24 hours with or without 1 μM etoposide were separated into cytosolic and mitochondrial fractions. The release of cytochrome *c* (cyt *c*) was evaluated by immunoblotting of cytosolic versus mitochondrial extracts using a specific antibody. β-actin was used as a loading control for the cytosolic fractions.

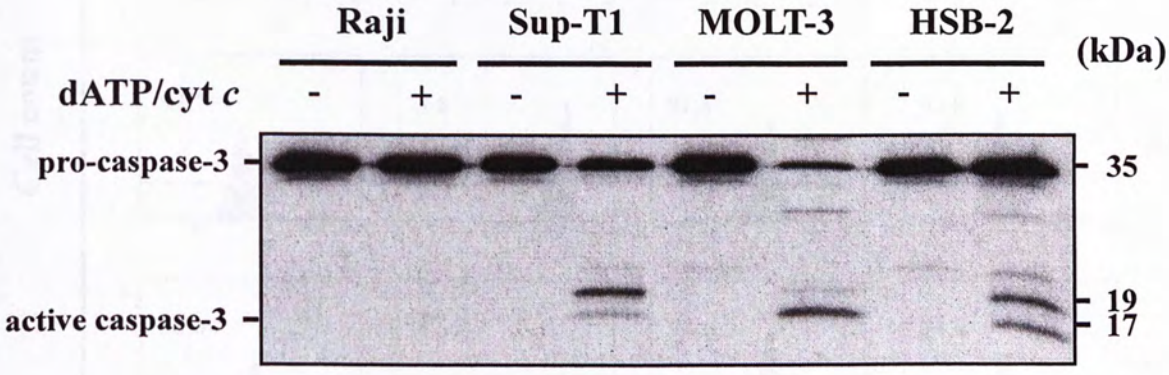

Fig. 3.13 Representative immunoblots showing the cytochrome *c*-dependent activation of pro-caspase-3 in leukemic cell lines.

S-100 cytosols were prepared from the leukemic cells, and exogenous dATP and cytochrome *c* (dATP/cyt *c*) were introduced to initiate caspase activation. Processing of pro-caspase-3 was determined by Western blotting.

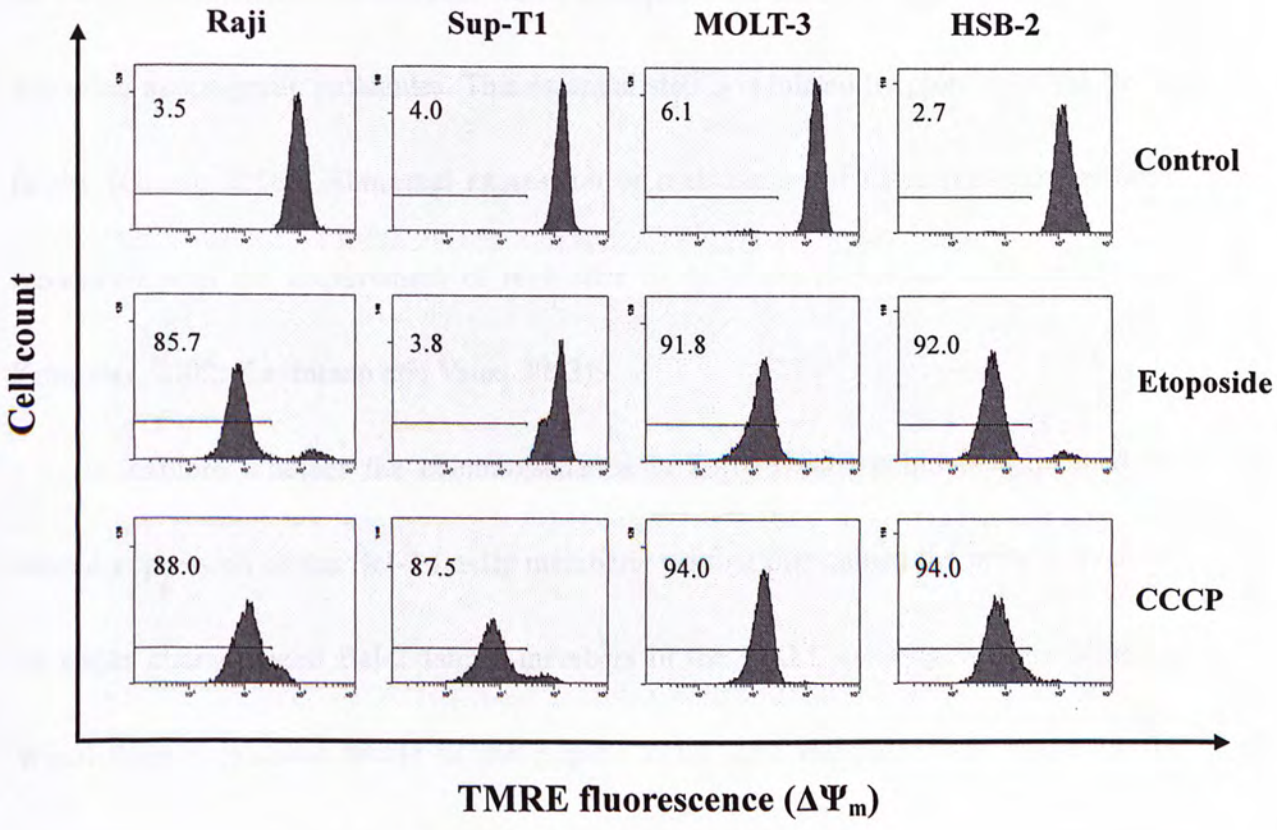

Fig. 3.14 **Representative histograms showing the etoposide-triggered dissipation of mitochondrial membrane potential in leukemic cell lines.**

Leukemic cells treated for 24 hours with or without 1 μM etoposide were subjected to flow cytometric analysis of mitochondrial membrane potential using a potential-sensitive probe, TMRE. CCCP was used as a positive control for membrane depolarization. The percentages of cells with reduction in mitochondrial membrane potential are shown.

3.4 Bim_{EL} is required for etoposide-induced apoptosis in Sup-T1 cells

Apoptosis that proceeds via the mitochondrial pathway involves permeabilization of the outer mitochondrial membrane, which is responsible for the release of cytochrome *c* and other apoptogenic molecules. This essential step is regulated by proteins of the Bcl-2 family (Green, 2006). Abnormal expression or malfunction of these proteins has been associated with the acquirement of resistance to apoptosis in tumour cells (Igney and Krammer, 2002; Kaufmann and Vaux, 2003).

To explore whether the chemoresistance in Sup-T1 cells could be explained by altered expression of the Bcl-2 family members, we first determined the protein levels of 10 better characterized Bcl-2 family members in the T-ALL cells by Western blotting. When their expression levels in the Sup-T1 cells were compared with those in the etoposide-sensitive HSB-2 and MOLT-3 cells, a marked elevation of the anti-apoptotic Bcl-2 was observed (Fig. 3.15). Moreover, expression levels of the pro-apoptotic member Bax and the extra-long isoform of pro-apoptotic Bim (Bim_{EL}) were apparently reduced in the Sup-T1 cells (Fig. 3.15). However, the levels of anti-apoptotic members Bcl-x_L and Mcl-1, as well as the pro-apoptotic members Bak, Bad, Bik, Bid and Nip-3 in Sup-T1 cells were not differed from the etoposide-sensitive T-ALL cells (Fig. 3.15). Hence, it appeared that caspase activation and apoptosis in the Sup-T1 cell line might be impeded at least partially by elevated level of Bcl-2 and subthreshold levels of Bax and Bim_{EL}.

We next considered if the altered expression levels of Bcl-2, Bax and Bim_{EL} render the Sup-T1 cells resistant to the etoposide-induced apoptosis. To address this question, we investigated whether silencing of Bcl-2 as well as enforced expression of Bax or Bim_{EL} could restore sensitivity to etoposide-induced apoptosis in the Sup-T1 cells. Results from the gene silencing experiments showed that transfection of a Bcl-2 antisense oligonucleotide into the Sup-T1 cells significantly reduced the protein level of Bcl-2 (Fig. 3.16 a, *lane 1 vs lane 3*), but not in the cells transfected with the corresponding, missense oligonucleotide (Fig. 3.16 a, *lane 2*). However, the reduced level of Bcl-2 could not resensitize the Sup-T1 cells to the etoposide-induced caspase-3 activation (Fig 3.16 b, *lane 4 vs lane 5*) and the consequent apoptosis, as judged by PARP cleavage (Fig. 3.16 c, *lane 4 vs lane 5*). Transient transfection of a wild-type Bax expression vector into the Sup-T1 cells had elevated the protein level of Bax (Fig. 3.17 a, *lane 1 vs lane 3*). Despite slight increases in the levels of active caspase-3 (p17; Fig. 3.17 b, *lane 4 vs lane 5*) and cleaved PARP (p89; Fig. 3.17 c, *lane 4 vs lane 5*) could be detected in the Bax-overexpressing cells upon the etoposide treatment, the levels of these fragments, however, were not significantly different from the cells transfected with an empty vector (Fig. 3.17 b and c, *lane 2*) or a Bax expression vector (Fig. 3.17 b and c, *lane 3*). This suggested that an enhanced expression of Bax still could not restore the etoposide-induced apoptosis in the Sup-T1 cells. We then tested the ability of

overexpressed, wild type Bim_{EL} to restore sensitivity of the Sup-T1 cells to the etoposide-induced apoptosis. Transient transfection of a Bim_{EL} expression vector into the Sup-T1 cells resulted in an elevation of Bim_{EL} protein levels (Fig. 3.18 a, *lane1 vs lane 3*) that was not observed in the cells transfected with an empty vector (Fig. 3.18 a, *lane1 vs lane 2*). Indeed, overproduction of Bim_{EL} in the Sup-T1 cells resulted in an elevated level of apoptosis, indicated by increased processing of pro-caspase-3 (Fig. 3.18 b, *lane2 vs lane 3*) and PARP (Fig. 3.18 c, *lane2 vs lane 3*). Strikingly, the p17 active subunit of caspase-3 and the p89 cleaved fragment of PARP were most readily detected in the Bim_{EL}-overexpressing cells following the etoposide treatment (Fig. 3.18 b and c, *lane 5*), indicating that the enhanced level of Bim_{EL} could resensitize the Sup-T1 cells to the etoposide-induced apoptosis. Taken together, these data clearly reveal a critical role of Bim_{EL} in determining the sensitivity to etoposide-induced apoptosis in Sup-T1 cells.

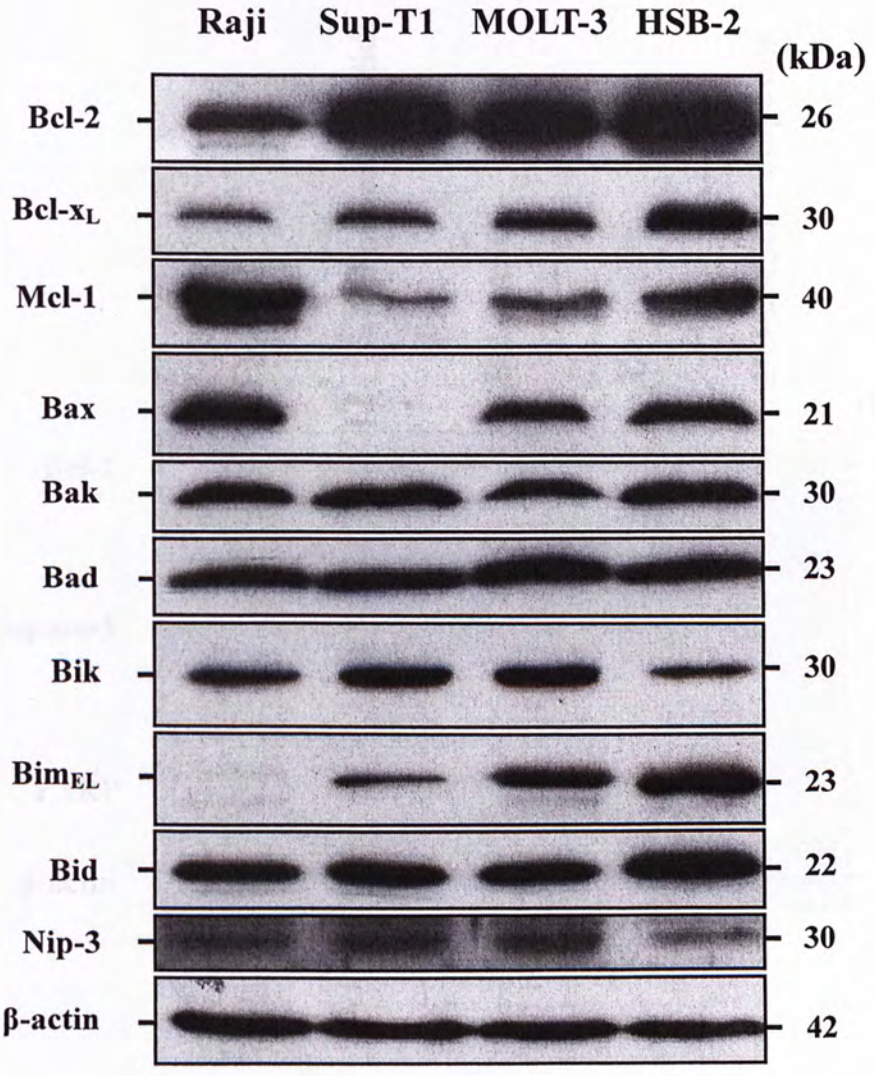

Fig. 3.15 **Representative immunoblots showing the expression levels of Bcl-2 family proteins in leukemic cell lines.**

Lysates from the leukemic cells were analyzed for protein levels of 10 Bcl-2 family members by Western blotting using specific antibodies. β-actin was used as a loading control.

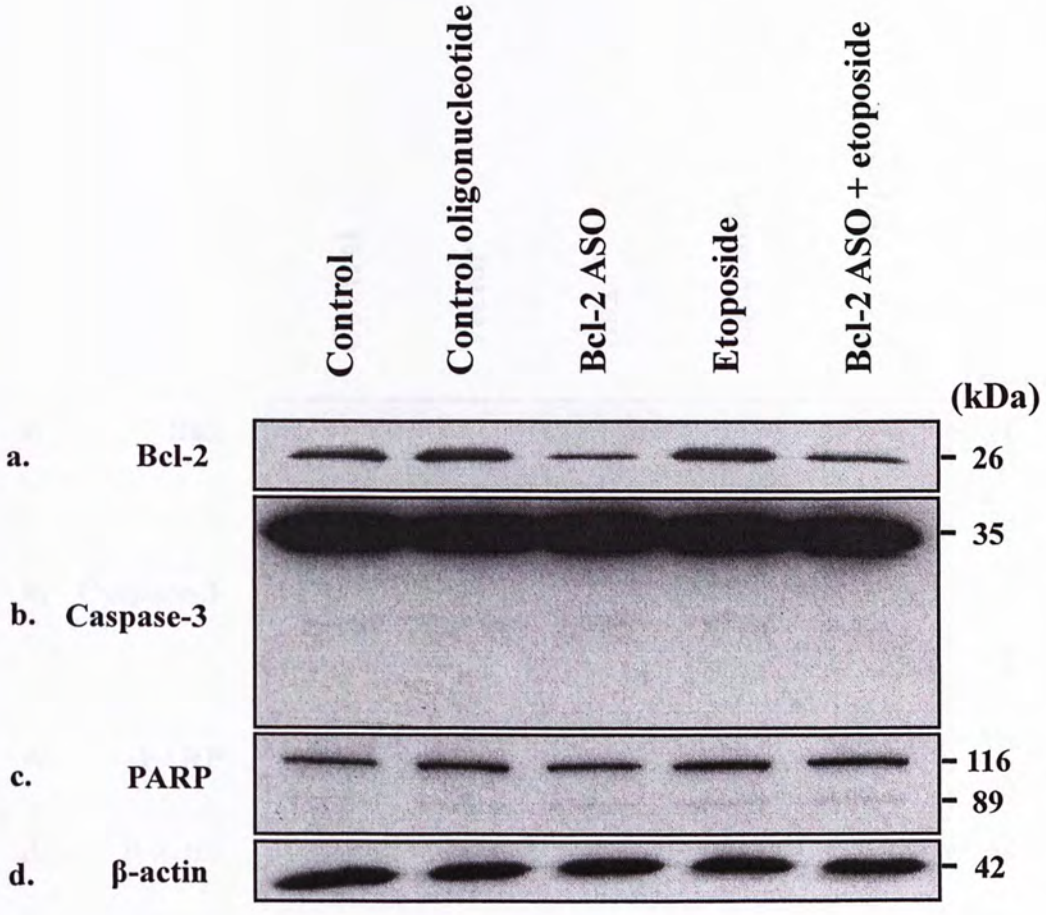

Fig. 3.16 **Representative immunoblots showing the effect of Bcl-2 silencing on the etoposide-induced apoptosis in Sup-T1 cells.**

Sup-T1 cells were either mock-transfected (*lane 1*) or transfected with a Bcl-2-specfic antisense oligonucleotide (*lane 3*), and treated with 1 μM etoposide for 24 hours (*lanes 4 and 5*). After treatment, cells were harvested, and the lysates were analyzed for Bcl-2 level as well as processing of pro-caspase-3 and PARP by Western blotting. Cells transfected with a control oligonucleotide (*lane 2*) that minimally reduced Bcl-2 expression were included as a negative control. β-actin was used as a loading control.

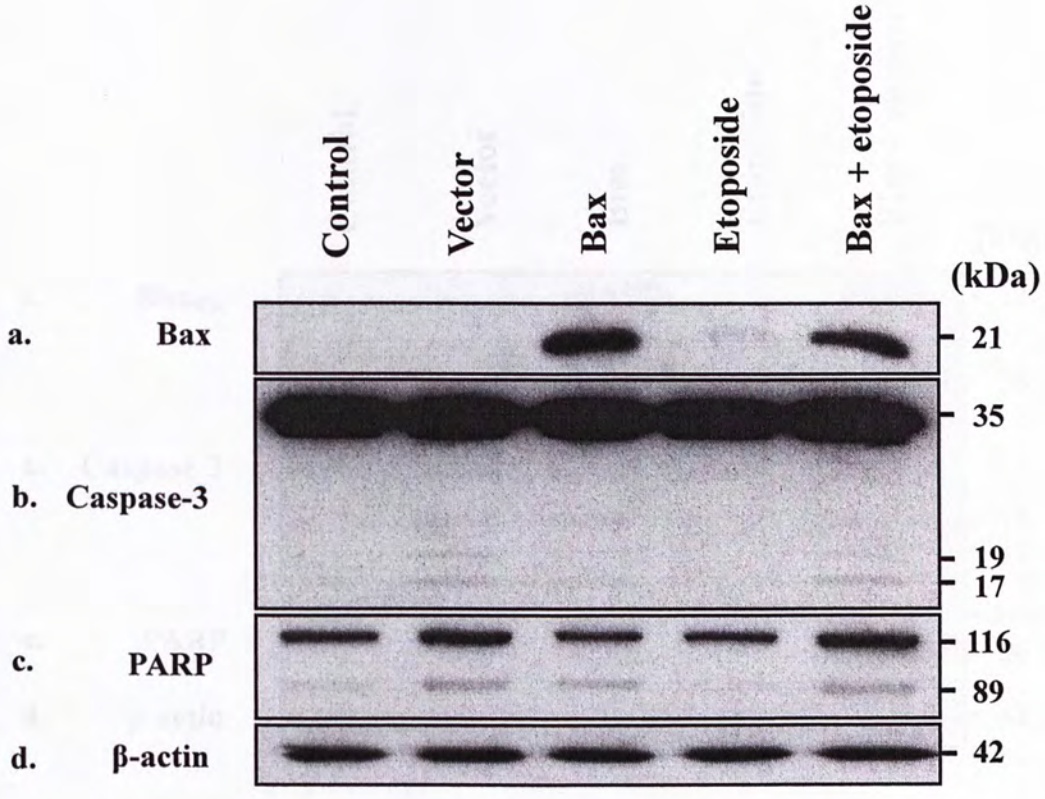

Fig. 3.17 **Representative immunoblots showing the effect of Bax overexpression on etoposide-induced apoptosis in Sup-T1 cells.**
Sup-T1 cells were either mock-transfected (*lane 1*) or transfected with a wild-type Bax expression vector (*lane 3*), and treated with 1 μM etoposide for 24 hours (*lanes 4 and 5*). After treatment, the cells were harvested, and the lysates were analyzed for Bax level as well as processing of pro-caspase-3 and PARP by Western blotting. The cells transfected with an empty vector (*lane 2*) were included as a negative control. β-actin was used as a loading control.

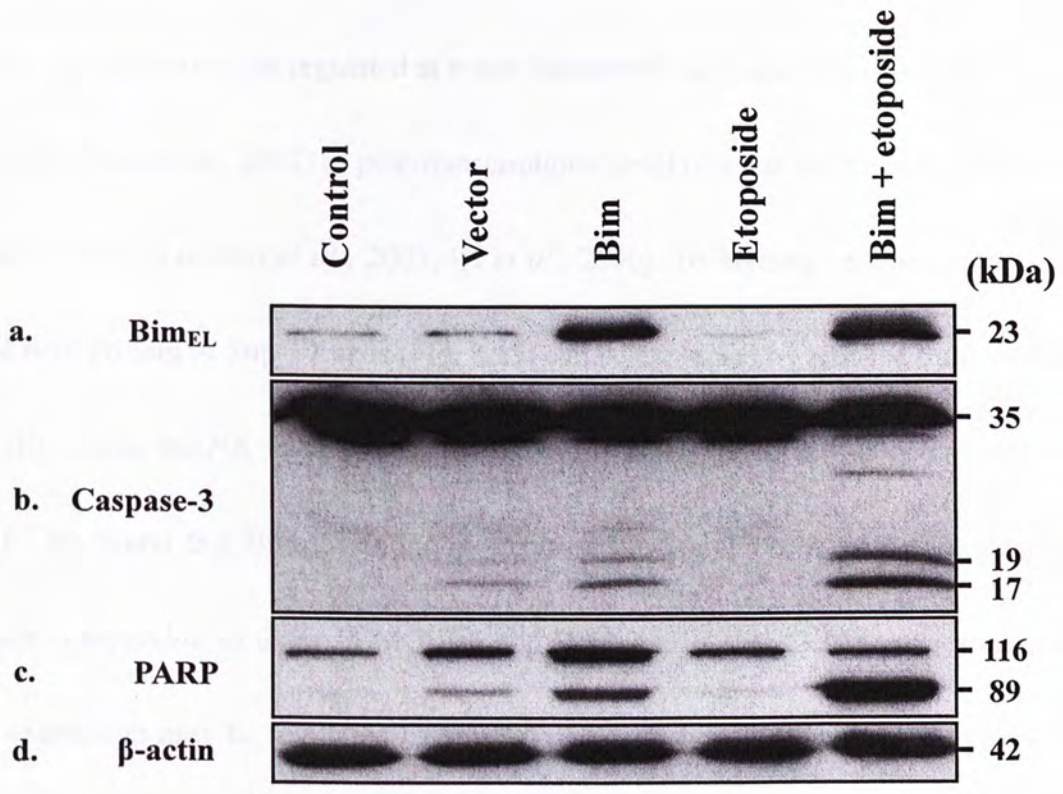

Fig. 3.18 **Representative immunoblots showing the effect of Bim$_{EL}$ overexpression on etoposide-induced apoptosis in Sup-T1 cells.**
Sup-T1 cells were either mock-transfected (*lane 1*) or transfected with a wild-type Bim$_{EL}$ expression vector (*lane 3*), and treated with 1 μM etoposide for 24 hours (*lanes 4 and 5*). After treatment, the cells were harvested, and the lysates were analyzed for Bim$_{EL}$ level as well as processing of pro-caspase-3 and PARP by Western blotting. The cells transfected with an empty vector (*lane 2*) were included as a negative control. β-actin was used as a loading control.

3.5 The reduced level of Bim_{EL} in Sup-T1 cells is owing to the presence of constitutively active JNK

Bim expression can be regulated at either transcriptional (Dijkers *et al.*, 2000; Putcha *et al.*, 2001; Stahl *et al.*, 2002) or post-transcriptional level (Biswas and Greene, 2002; Lei and Davis, 2003; Luciano *et al.*, 2003; Qi *et al.*, 2006). To investigate whether the low level of Bim protein in Sup-T1 cells (Fig. 3.15) can be explained by reduced transcription of the Bim gene, mRNA level corresponding to the Bim_{EL} isoform was determined by RT-PCR. We found that Bim_{EL} was highly expressed in Sup-T1 cells, and its expression level was comparable to those in MOLT-3 and HSB-2 cells (Fig. 3.19), suggesting that Bim_{EL} expression may be regulated by some post-transcriptional mechanisms in Sup-T1 cells. The mRNA levels of Bcl-2 and Bax in Sup-T1 cells, on the contrary, were found similar to those of their respective protein products (Fig. 3.15 and 3.19), indicating that the expression levels of these two proteins are determined transcriptionally.

The proteosome pathway has been shown previously to be involved in the degradation of Bim following its phosphorylation (Luciano *et al.*, 2003; Qi *et al.*, 2006). Thus, the apparent low level of Bim_{EL} protein in Sup-T1 cells may possibly be resulted from the same pathway. To address this question, Sup-T1 cells were treated in the presence or absence of the proteosome inhibitor, MG-132. Western blot analysis revealed that Bim_{EL} could hardly be detected in the absence of proteosome inhibitor (Fig. 3.20,

lane 1). Incubation with MG-132, however, significantly increased the protein level of Bim_{EL} in Sup-T1 cells (Fig. 3.20, *lane 2*). In addition, treatment with proteosome inhibitor resulted in mobility shift of the Bim_{EL} protein, evidenced by the presence of immunoreactive Bim_{EL} with higher molecular masses (Fig. 3.20, *lane 2*). To determine whether the observed mobility shift was due to phosphorylation of the Bim_{EL} protein, the cell lysate from Sup-T1 cells treated with the proteosome inhibitor was digested with calf intestinal alkaline phosphatase (CIAP). Digestion with CIAP resulted in a partial increase in the mobility of Bim_{EL} species (Fig. 3.20, *lane 3*), when compared with the undigested sample (Fig. 3.20, *lanes 2*). Taken together, these data suggest that the newly synthesized Bim_{EL} protein in Sup-T1 cells is phosphorylated and form different species. This serves as a signal for degradation through the proteosome pathway, thereby resulting in the loss of its expression.

Bim has been reported to be a target for ERK (Ley *et al.*, 2003; Luciano *et al.*, 2003), JNK (Lei and Davis, 2003; Putcha *et al.*, 2003), p38 (Cai *et al.*, 2006) and Akt phosphorylation (Qi *et al.*, 2006). To investigate whether these protein kinases are also responsible for modulation of Bim_{EL} expression in Sup-T1 cells, we first determined their activities by using antibodies that only recognize the phosphorylated, active forms of these kinases. Western blot analysis showed that all of these kinases were highly expressed in the Sup-T1 cells (Fig. 3.21). However, only Akt and JNK were found

constitutively active in the Sup-T1 cells (Fig. 3.21). To explore the potential roles of these protein kinases in Bim_{EL} degradation, Sup-T1 cells treated with specific inhibitors of the ERK (PD98059, U0126), JNK (SP600125), p38 (SB203580) and Akt (LY294002, wortmannin) pathways were subjected to detection of endogenous Bim_{EL} by Western blotting. We found that the expression of Bim_{EL} could not be restored by the PI3-K inhibitor, LY294002 or wortmannin. Similar results were obtained from the cells treated with the MEK inhibitor, PD98059 or U0126, or the p38 inhibitor SB203580 (Fig. 3.22). Surprisingly, treatment of Sup-T1 cells with the JNK inhibitor SP600125 significantly elevated the Bim protein level (Fig. 3.22), suggesting that the degradation of Bim_{EL} in Sup-T1 cells is mediated by JNK-dependent events.

Bim_{EL} was found crucial for the etoposide-induced apoptosis in Sup-T1 cells (Fig. 3.18). Then, suppression of its expression by some JNK-mediated events is very likely to be the reason that renders this cell line resistant to the etoposide-induced apoptosis. To address this question, we investigated whether the JNK inhibitor SP600125 could resensitize Sup-T1 cells to the etoposide-induced apoptosis. The results revealed that the JNK activity in Sup-T1 cells was reduced by the SP600125 treatment, indicated by the decreased level of active, phosphorylated c-Jun (Fig. 3.23 a, *lane 1 vs lane 2*). Again, Bim_{EL} expression was significantly elevated when the JNK activity was inhibited (Fig 3.23 c, *lane 1 vs lane 2*), but the increased level of Bim_{EL} did not result in caspase-3

activation and PARP cleavage (Fig 3.23 d and e, *lane 2*). However, the p17 active subunit of caspase-3 and cleaved fragment of PARP were readily detected in cells preincubated with SP600125 followed by etoposide treatment (Fig. 3.23 d and e, *lane 3 vs lane 4*), indicating that suppression of JNK activity can resensitize Sup-T1 cells to the etoposide-induced apoptosis. Taken together, our results indicate that the resistance to etoposide-induced apoptosis in Sup-T1 cells is due to the presence of constitutively JNK.

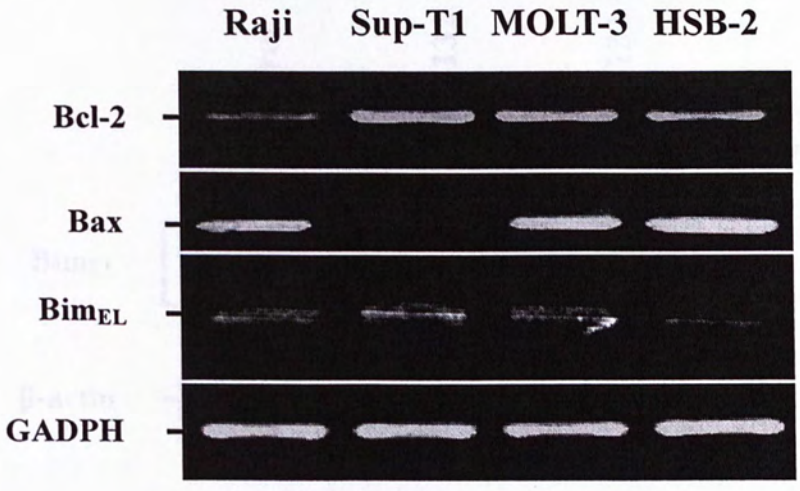

Fig. 3.19 **Representative images showing the mRNA levels of Bcl-2, Bax and Bim in leukemic cell lines.**

Total mRNA was extracted from the leukemic cells, and the mRNA levels of Bcl-2, Bax and Bim_{EL} were assessed by RT-PCR. GADPH was used as a loading control.

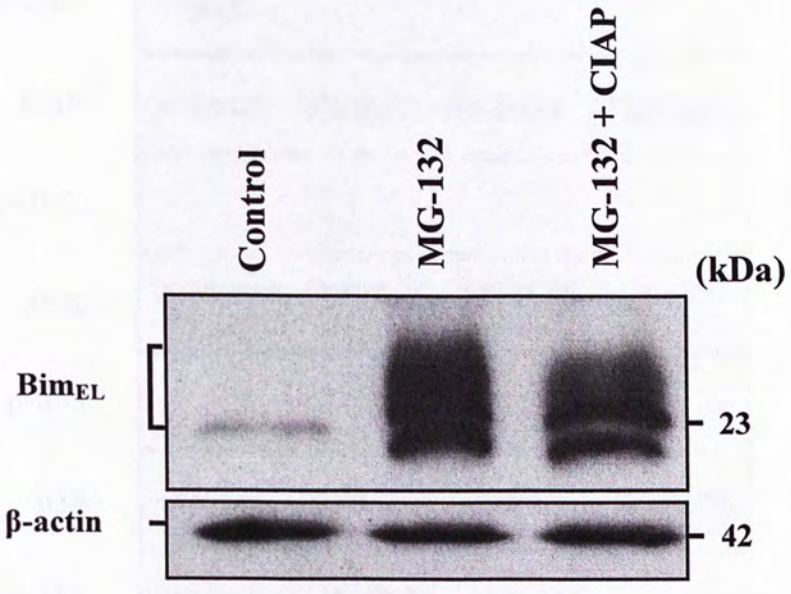

Fig. 3.20 **Representative immunoblots showing the effect of proteosome inhibitor on Bim expression in Sup-T1 cells.**

Sup-T1 cells were cultured for 24 hours in the absence (*lane 1*) or presence of 50 μM proteosome inhibitor, MG-132 (*lane 2*). After treatment, the cells were harvested, and the lysates were analyzed for Bim$_{EL}$ protein level by Western blotting. Aliquot of the lysate from cells treated with MG-132 was digested with calf intestinal alkaline phosphatase (CIAP, *lane 3*) for 1 hour, prior to the Western blot analysis. β-actin was used as a loading control.

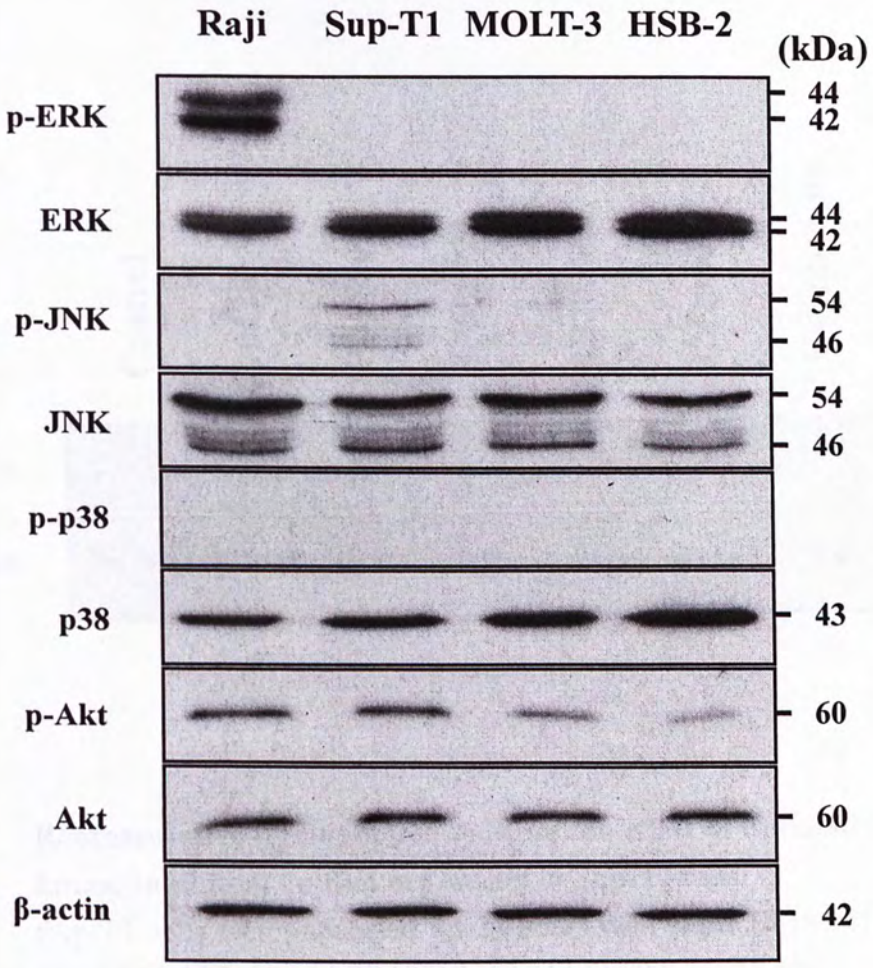

Fig. 3.21 **Representative immunoblots showing the activities of protein kinases in leukemic cell lines.**

Phosphorylated and active forms of ERK, JNK, p-38 and Akt in the leukemic cells were determined by Western blotting using phospho-specific antibodies. Blots were also analyzed for the total protein levels of each kinase. β-actin was used as a loading control.

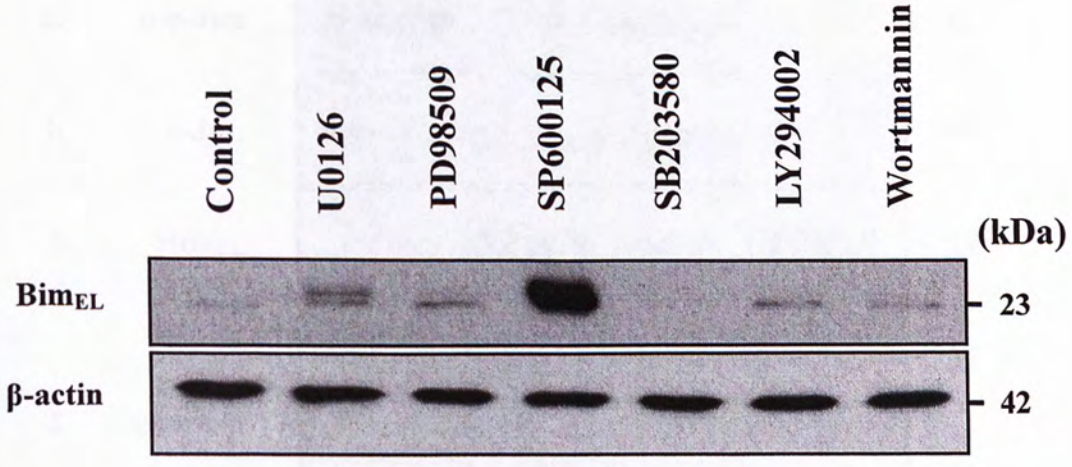

Fig. 3.22 Representative immunoblots showing the effect of different protein kinase inhibitors on Bim expression in Sup-T1 cells.

Sup-T1 cells were incubated for 24 hours with either DMSO (*control*), 10 μM U0126, 10 μM PD98509, 10 μM SP600125, 15 μM SB203580, 50 μM LY294002, or 100 nM wortmannin. The cell lysates were analyzed for Bim protein levels by Western blotting. β-actin was used as a loading control.

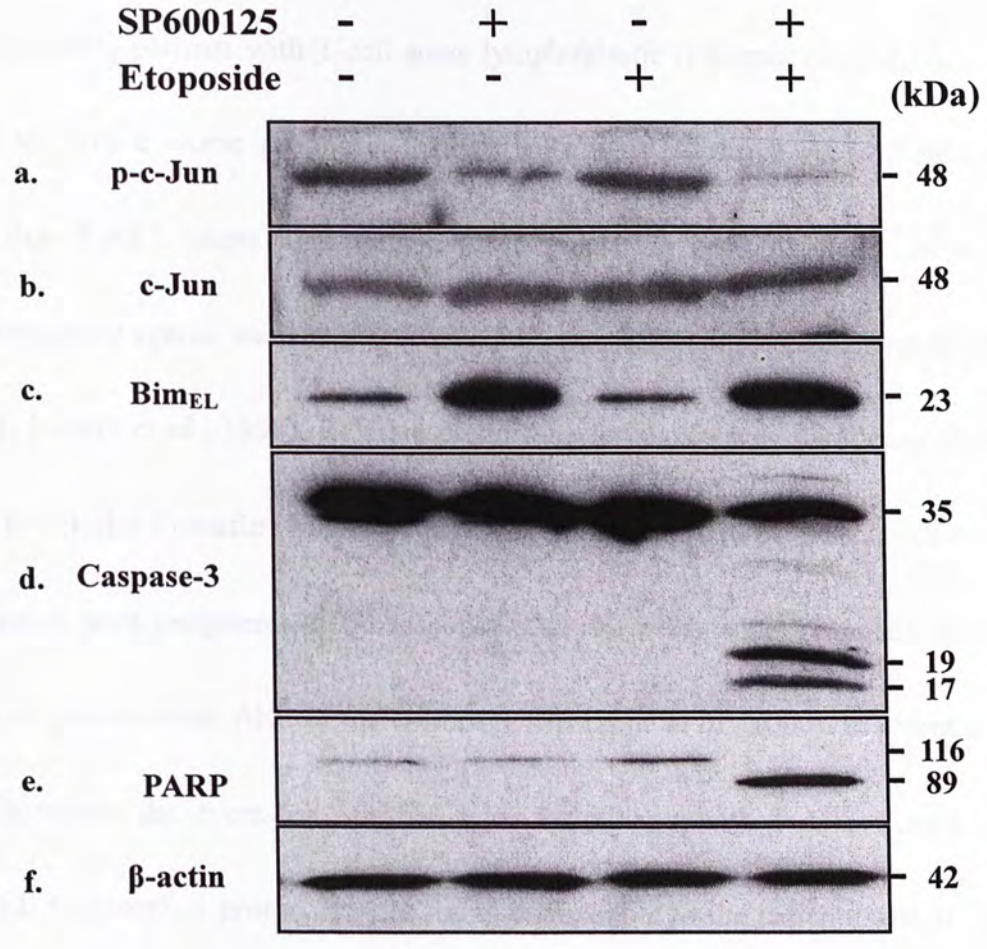

Fig. 3.23 **Representative immunoblots showing the effect of JNK inhibitor on etoposide-induced apoptosis in Sup-T1 cells.**

Sup-T1 cells were preincubated in the absence (*lane 1*) or presence (*lane 2*) of 10 μM JNK inhibitor, SP600125, for one hour and then treated with (*lane 4*) or without (*lane 3*) 1 μM etoposide for 24 hours. The inhibitor remained present throughout the etoposide treatment. The cell lysates were then analyzed for p-c-Jun, c-Jun and Bim$_{EL}$ levels as well as processing of pro-caspase-3 and PARP by Western blotting. β-actin was used as a loading control.

Chapter 4 Discussion

Historically, patients with T-cell acute lymphoblastic leukemia (T-ALL) have been reported to have a worse prognosis than patients with B-lineage ALL. Pieters *et al.* showed that T-ALL blasts have demonstrated greater *in vitro* resistance to standard chemotherapeutic agents, such as glucocorticoids, vincristine, and asparaginase (Pieters *et al.*, 1993; Pieters *et al.*, 1998). Relative prednisone resistance was also demonstrated *in vivo* on the Berlin-Frankfurt-Muenster protocol, in which 36% of T-ALL patients had demonstrated poor peripheral blood response after the prednisone treatment, compared with 5% of patients with ALL of the B-lineage (Schrappe *et al.*, 2000). In recent clinical studies, however, the event-free survival rates for patients with T-ALL treated on the DFCI ALL Consortium protocols were found comparable to the patients with B-lineage ALL (Silverman *et al.*, 2000). Unique features of these protocols that might have contributed to the improved outcome of T-ALL patients include consolidation with doxorubicin and weekly high-dose asparaginase, as well as frequent pulses of high-dose corticosteroids.

Despite the adverse risk associated with T-lineage ALL has progressively been overwhelmed by intensive chemotherapeutic regimens, approximately 20% of the patients with T-ALL continue to fail the therapy. Higher rate of induction failure and early relapse

in these patients suggest that their blast cells are more inherently resistant to conventional chemotherapeutic agents. Further augmentation of the currently used intensive chemotherapeutic regimens may not be warranted because of the likelihood of significant adverse effects. Thus, the identification of additional prognostic variables and chemoresistance mechanisms that can be used for more precise tailored-therapy is of top priority. Evaluation of the underlying molecular mechanisms determining response or resistance not only enables clinicians to define prognostic markers, but also facilitates the design of molecularly targeted agents potentially reversing the causative lesion.

Unfortunately, the cause of treatment failure in T-ALL patients is still largely unknown. Only a few candidate genes have been implicated in determining the sensitivity of T-ALL cells to chemotherapeutic agents. Steinbach *et al.* showed that higher level of multidrug resistance-associated protein 3 (MRP-3) in T-ALL patients is associated with a poor *in vivo* response to prednisone (Steinbach *et al.*, 2003). Ramakers-van Woerden *et al.* demonstrated that p16 homozygously deleted T-ALL cases have a significant worse disease-free survival. However, the p16 gene deletion was only correlated with the *in vitro* resistance to dexamethasone and prednisolone, among the 10 drugs tested (Ramakers-van Woerden *et al.*, 2001). A more recent study showed that a T-ALL cell line, MOLT-4, having aberrant Notch-1 signaling is chemoresistant (Mungamuri *et al.*, 2006).

Aberrations in apoptotic pathways have been detected in a variety of cancers,

suggesting that loss of the ability of cells to undergo apoptosis may contribute to carcinogenesis. Because cancer therapies such as radiation, glucocorticoids, and chemotherapeutic drugs exert their beneficial effects, at least in part, by inducing apoptosis in the cancer cells, the same aberrations in apoptotic pathways would be predicted to contribute to resistance (Kaufmann and Vaux, 2003). In the present study, we therefore speculated that defective apoptosis could render T-ALL cells resistant to chemotherapeutic agents.

By studying the cellular responses to an apoptogenic drug, etoposide, we identified an apoptosis-resistant T-ALL cell line, Sup-T1. Thus, we recruited Sup-T1 as a model cell line to study the possible mechanisms of resistance to etoposide-induced apoptosis in T-ALL cells. Flow cytometric analysis revealed that etoposide treatment apparently induced cell cycle arrest in Sup-T1 cells. However, cell cycle arrest and apoptosis are not sequential events in the Sup-T1 model, implying that resistance to etoposide in Sup-T1 cells is linked to defective apoptosis but not events associated with the cell cycle. Further experiments showed that Sup-T1 cells were deficient in the etoposide-induced activation of caspases and mitochondrial cytochrome *c* release (Fig. 3.10, 3.11 and 3.12). However, addition of exogenous cytochrome *c* in the cell-free apoptosis reactions induced prominent caspase-3 activation (Fig. 3.13). These findings suggest that the chemoresistance observed in Sup-T1 cells is due to its insusceptibility to drug-induced

mitochondrial alterations.

Proteins in the Bcl-2 family are the central regulators of apoptosis. Abnormal expressions of these Bcl-2 family proteins have been implicated in the development of chemoresistance in tumor cells. Overexpressions of anti-apoptotic members Bcl-2, Bcl-x_L and Mcl-1 have been shown to confer resistance to a variety of anticancer agents. Besides, tumors can also acquire resistance by downregulating expressions of the proapoptotic members Bax and Bid (Igney and Krammer, 2002). Analysis of expression of the Bcl-2 family proteins in this study revealed that the levels of Bax, Bcl-2 and Bim in Sup-T1 cells were differed from those in the etoposide-sensitive T-ALL cell lines (Fig. 3.15). Indeed, our data obtained in the Sup-T1 model show that the defective apoptosis and activation of caspases are linked to a lack of expression of Bim, a pro-apoptotic member of the Bcl-2 family proteins. Transient transfection of Bim_{EL} cDNA into resistant Sup-T1 cells yielded elevated level of Bim protein and restored their sensitivity to the etoposide-induced apoptosis (Fig. 3.18). This provides direct evidence that Bim is an important determinant for the sensitivity of T-ALL Sup-T1 cells to the etoposide-induced apoptosis.

Bim, the Bcl-2 interacting mediator of cell death, is expressed by a wide variety of tissues, but is most prominently expressed by cells of hematopoietic origin (O'Reilly *et al.*, 2000). Although multiple Bim mRNA transcripts have been described that are

generated by alternative splicing (O'Conner *et al.*, 1998; Bouillet *et al.*, 2001; Marani *et al.*, 2002), most tissues express one predominant isoform of Bim as determined by Western blotting analysis, termed Bim_{EL}. Several lines of evidence have shown that Bim is critical for apoptosis in certain types of cells. Gene-knockout study revealed that Bim is a principal regulator of hematopoietic homeostasis (Bouillet *et al.*, 1999). In its absence, leukocyte numbers rise and plasma-cell accumulation provokes the onset of an autoimmune disease. This onset occurs because Bim is essential for the elimination of autoreactive lymphocytes by apoptosis (Bouillet *et al.*, 2002). Putcha *et al.* demonstrated that induction of Bim is also critical for apoptosis in neurons (Putcha *et al.*, 2001). Moreover, Bim is required for apoptosis induced by paclitaxel, dexamethasone, and histone deacetylase inhibitors (Strasser, 2005; Tan *et al.*, 2005; Zhao *et al.*, 2005). In T-ALL cells, Bim induction was found essential for glucocorticoid-induced apoptosis (Lu *et al.*, 2006). In the current study, we provide the first evidence showing that lack of Bim expression could render T-ALL cells resistant to the etoposide-induced apoptosis.

Under conditions that promote cell growth, Bim is bound to dynein light chain LC8 of the microtubular motor complex, and is sequestered away from other Bcl-2 family members (Puthalakath *et al.*, 1999). Following a pro-apoptotic stimulus, however, Bim localizes to the mitochondria, where it initiates the mitochondrial cell death pathway by either directly activating Bax-like proteins (Marani *et al.*, 2002; Liu *et al.*, 2003) or by

binding to the pro-survival Bcl-2 family members and thereby releasing the Bax-like proteins (Terradillos *et al.*, 2002; Yamaguchi and Wang, 2002; Wilson-Annan *et al.*, 2003). As silencing of Bcl-2 did not resensitize Sup-T1 cells to the etoposide-induced apoptosis (Fig. 3.16), and Sup-T1 cells do not express Bax (Fig. 3.15), we believe that the levels of Bim in Sup-T1 cells is insufficient to neutralize the anti-apoptotic actions of Mcl-1 or Bcl-x_L, thereby hindering the release of mitochondrial cytochrome *c* and the consequent apoptotic events.

Expression levels of BH3-only proteins are key determinants of cellular survival and are therefore under stringent control. Transcriptional and post-translational regulatory mechanisms have previously been described in the control of Bim expression levels. Transcriptional control of Bim appears to be complex, as different groups have reported different modes of regulation. Growth factor withdrawal-induced up-regulation of Bim expression was shown to require JNK activation in neurons (Harris and Johnson, 2001; Putcha *et al.*, 2001) but depended on the forkhead transcription factor FKHR-L1 in hematopoetic cells (Dijkers *et al.*, 2000). Other studies with hematopoetic cell lines have indicated that cytokine stimulation represses Bim expression through activation of the mitogen-activated protein kinase pathway or the phosphatidylinositol 3' kinase (Shinjyo *et al.*, 2001). However, the lack of Bim expression in Sup-T1 cells is not likely regulated transcriptionally, as significant level of Bim mRNA was detected (Fig. 3.19), suggesting

that the low level of Bim protein in Sup-T1 cells is a consequence of post-transcriptional events.

Ubiquitin-mediated degradation of Bim through the proteasomal pathway has recently emerged as a post-translational mechanism controlling cellular Bim level. In the different cellular models studied, the loss of Bim protein could be blocked by proteosome inhibitor MG-132 (Luciano *et al.*, 2003). Furthermore, multiple ubiquitinated forms of Bim protein were demonstrated in the presence of proteosome inhibitors (Akiyama *et al.*, 2003), implicating the involvement of ubiquitin-proteosomal pathway in Bim degradation. In agreement with these studies, we showed that the level of Bim_{EL} in Sup-T1 cells was significantly restored after incubation with MG-132 (Fig. 3.20). Thus, the newly synthesized Bim_{EL} in Sup-T1 cells is rapidly targeted to the proteosome and degraded, therefore resulting in the lack of its expression.

By Western blotting, multiple bands of immunoreactive Bim_{EL} species were detected in Sup-T1 cells following treatment with MG-132, and at least two of which were sensitive to phosphatase digestion (Fig. 3.20), suggesting that Bim_{EL} had been phosphorylated at multiple sites. This finding is in line with the results of two previous studies in which the number of BimEL species was examined by two-dimensional gel electrophoresis (Seward *et al.*, 2003; Ley *at al.*, 2004). For example, it was shown that the Bim_{EL} immunoprecipitated from ^{32}P-labeled murine HT-2 or Bal17 lymphocytes could be

separated into at least four ^{32}P-labeled forms (Seward *et al.*, 2003). Similar results were obtained in CCL39 fibroblasts, where addition of the fetal bovine serum to serum-starved cells resulted in the appearance of three phosphorylated forms of immunoreactive Bim_{EL} (Ley *at al.*, 2004). Of note, our data demonstrated that some of the Bim_{EL} species with higher molecular mass were not sensitive to phosphatase digestion (Fig.3.20). These data imply that, apart from phosphorylation, Bim_{EL} is also subjected to other types of modification that targeting it to the proteosome.

The role of the ERK pathway in Bim phosphorylation has been intensively investigated. The fetal bovine serum-induced appearance of multiple forms of Bim_{EL} in CCL39 cells was shown to be sensitive to the ERK1/2 pathway inhibitor, PD184352, suggesting that multiple ERK phosphorylation sites exist in Bim_{EL} (Ley *et al.*, 2004). Studies in the IL-3-dependent murine pro-B cell line, FL5.12, demonstrated that when IL-3 was added back to the cytokine-deprived cells, there was a rapid shift in the mobility of Bim_{EL} to higher molecular weight forms (Harada *et al.*, 2004). Further, six Bim_{EL} mutants were generated by alanine substitution of putative ERK phosphorylation sites, and tested for their ability to be phosphorylated *in vivo*. Three mutants, S55A, S65A, and S100A, failed to undergo a mobility shift, indicating that ERK could potentially phosphorylate Bim_{EL} at these three sites. In our study, active, phosphorylated forms of ERK were not detected in Sup-T1 cells (Fig. 3.21). Furthermore, two specific MEK

inhibitors, PD98059 and U0126, failed to block degradation of Bim_{EL} (Fig. 3.22). Therefore, the ERK pathway is not likely to be responsible for phosphorylation of Bim_{EL} in the Sup-T1 model.

Likewise, a very recent study in an IL-3-dependent B-cell line demonstrated that Akt, a prosurvival kinase, is also capable to phosphorylate Bim_{EL} (Qi *et al.*, 2006). The IL-3-induced appearance of multiple, phosphorylated species of Bim_{EL} was partially blocked by the PI3-K inhibitor, LY294002. *In vitro* kinase assays showed that recombinant Akt could directly phosphorylate a GST-Bim_{EL} fusion protein (Qi *et al.*, 2006). However, our data suggest that Akt pathway is not responsible for the phosphorylation of Bim_{EL} in Sup-T1 cells. Although Akt is constitutively active (Fig. 3.21), addition of the PI3-K inhibitors, LY294002 and wortmannin, could not restore the Bim_{EL} level (Fig. 3.22).

In fact, we showed that the JNK pathway is necessary to promote Bim_{EL} phosphorylation and its turnover. Blockage of the JNK pathway by a specific inhibitor, SP600125, yielded elevated level of Bim_{EL} in the Sup-T1 cells (Fig. 3.22), suggesting that proteosomal degradation of Bim_{EL} requires, at least in part, an activated JNK pathway. Furthermore, Bim_{EL} species with higher molecular masses were no longer detected in the SP600125-treated cells (Fig. 3.22 and 3.23). Thus, phosphorylation of Bim_{EL} seems to be exclusively depended on the JNK pathway. JNK phosphorylation of Bim has been

demonstrated in fibroblasts (Lei and Davis, 2003) and neurons (Putcha *et al.*, 2003). In fibroblasts, JNK phosphorylation of Bim_L causes its release from the dynein motor complex, thereby allowing it to induce Bax-dependent apoptosis. In neurons, the mechanism by which JNK phosphorylation affects the pro-apoptotic activity of Bim_{EL} is still unclear. Nevertheless, evidence of Bim phosphorylation by JNK that has been documented so far is linked to the potentiation of apoptosis. Here we show for the first time that Bim_{EL} phosphorylation by the JNK pathway could plays an anti-apoptotic role by targeting Bim_{EL} for degradation via the proteosome. The different biological consequences of JNK phosphorylation of Bim_{EL} may have resulted from analysis of different types of tissues, perhaps indicating cell-type specific regulation of Bim. Of note, despite no known kinase downstream of JNK has been identified, it is not yet clear whether JNK itself is the kinase responsible for phosphorylating Bim_{EL}. Further studies should be directed to define the identity of the kinase and the sites of phosphorylation.

Numerous evidence shows that JNK functions as a pro-apoptotic kinase (Davis, 2003). However, a recent study revealed that JNK can also suppress apoptosis by phosphorylating the pro-apoptotic Bcl-2 family protein, Bad (Yu *et al.*, 2004). Thus, JNK can also function as an anti-apoptotic kinase. In this study, we demonstrate that the JNK axis can also suppress apoptosis in T-ALL Sup-T1 cells by phosphorylation of Bim_{EL}.

In conclusion, we have identified a novel mechanism of chemoresistance in T-ALL

cells (Fig. 4.1). Our findings also provide new insights into how the JNK pathway regulates Bim_{EL} level in leukemic cells. It will be of interest to determine in future studies whether the cellular Bim level is also associated with resistance to antileukemic agents in T-ALL patients. It is expected the findings of this study will help to develop novel, target-based strategies for the treatment of resistant T-ALL.

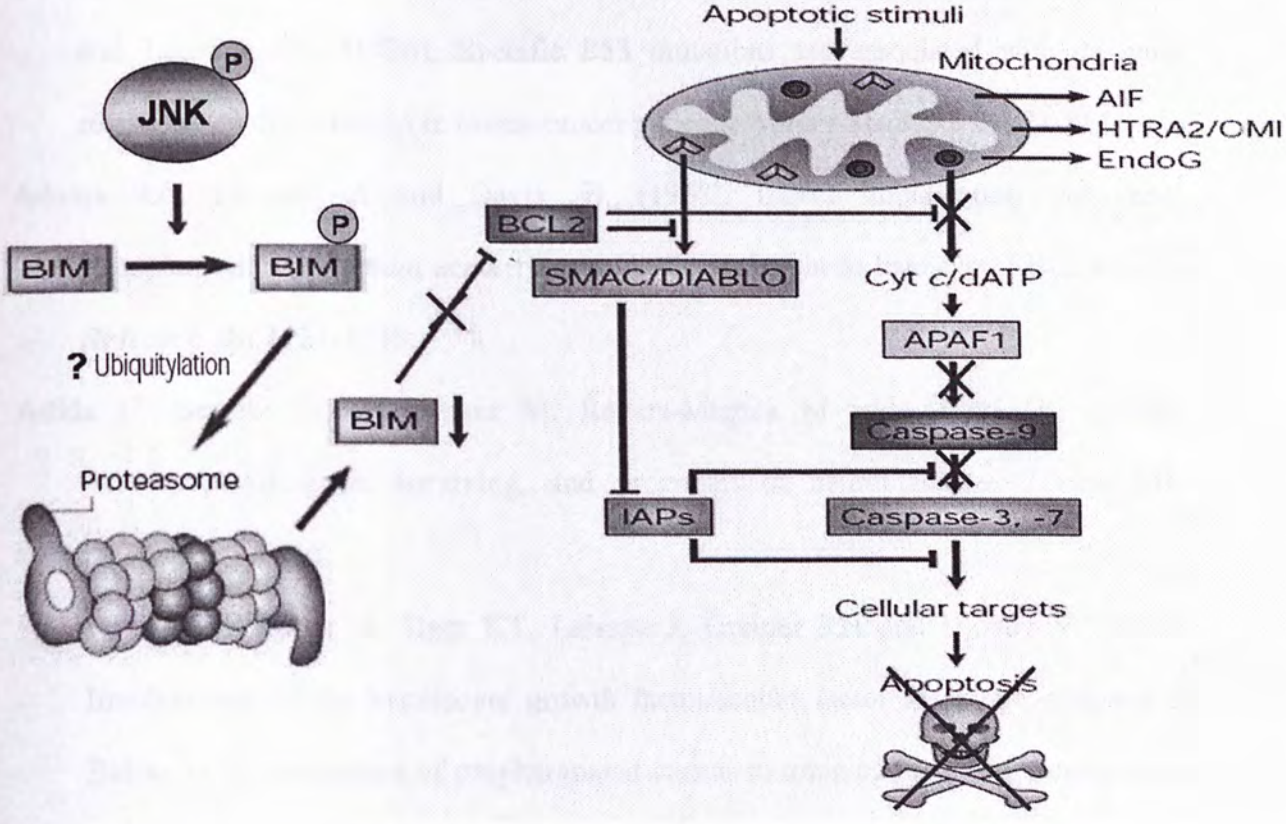

Fig. 4.1 **The apoptosis resistance mechanisms in Sup-T1 cells.**
Constitutively active JNK phosphorylates the newly synthesized Bim_{EL} in Sup-T1 cells. Phosphorylated Bim_{EL} is then targeted to the proteosome in which ubiquitination may be involved in this process. The proteosome then degrades Bim_{EL} so that its protein level is significantly reduced. The reduced Bim_{EL} level is not sufficient to inhibit the anti-apoptotic functions of the pro-survival Bcl-2 family proteins. Consequently, the release of mitochondrial cytochrome *c*, caspase activation and downstream apoptotic events are constantly blocked.

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